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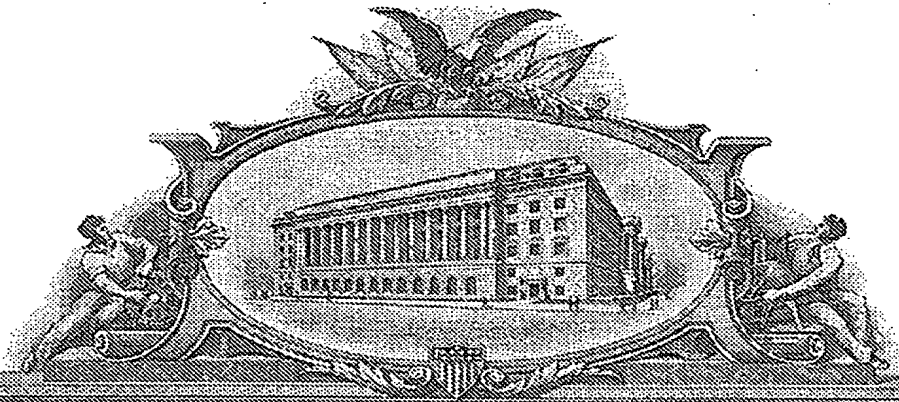
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ATTORNEY DOCKET NO. 21085.0123U1
Page 1 of 2

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 C.F.R. § 1.53(c).

		Docket Number	21085.0123U1	Type a Plus Sign (+) inside this box	+																		
INVENTOR(s)																							
LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (City and Either State or Foreign Country)																				
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TITLE OF INVENTION (500 characters max)																							
METHODS AND COMPOSITIONS FOR IDENTIFYING COMPOUNDS THAT INHIBIT HIV-1 SUBUNIT-SPECIFIC REVERSE TRANSCRIPTASE																							
CORRESPONDENCE ADDRESS																							
Janell T. Cleveland Customer Number 23859																							
ENCLOSED APPLICATION PARTS (Check All That Apply)																							
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%;"><input checked="" type="checkbox"/> Provisional Application Title Page</td> <td style="width: 20%; text-align: right;"><i>Number of Pages</i></td> <td style="width: 30%; text-align: right;">[01]</td> </tr> <tr> <td><input checked="" type="checkbox"/> Specification (includes Description, Claims, & Abstract)</td> <td style="text-align: right;"><i>Number of Pages</i></td> <td style="text-align: right;">[69]</td> </tr> <tr> <td><input checked="" type="checkbox"/> Drawing(s)</td> <td style="text-align: right;"><i>Number of Sheets</i></td> <td style="text-align: right;">[11]</td> </tr> <tr> <td><input checked="" type="checkbox"/> Sequence Listing (Paper Copy)</td> <td style="text-align: right;"><i>Number of Sheets</i></td> <td style="text-align: right;">[08]</td> </tr> <tr> <td colspan="3"><input checked="" type="checkbox"/> Authorization to Treat Reply Requesting Extension of Time as Incorporating Petition for Extension of Time</td> </tr> <tr> <td colspan="3"><input checked="" type="checkbox"/> Other (<u>Postcard</u>)</td> </tr> </table>						<input checked="" type="checkbox"/> Provisional Application Title Page	<i>Number of Pages</i>	[01]	<input checked="" type="checkbox"/> Specification (includes Description, Claims, & Abstract)	<i>Number of Pages</i>	[69]	<input checked="" type="checkbox"/> Drawing(s)	<i>Number of Sheets</i>	[11]	<input checked="" type="checkbox"/> Sequence Listing (Paper Copy)	<i>Number of Sheets</i>	[08]	<input checked="" type="checkbox"/> Authorization to Treat Reply Requesting Extension of Time as Incorporating Petition for Extension of Time			<input checked="" type="checkbox"/> Other (<u>Postcard</u>)		
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METHOD PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (Check One)	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR § 1.27.	FILING FEE AMOUNT \$ 80.00
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☒ Yes. The name of the U.S. Government agency and the Government contract number are:
National Institutes of Health Grant No. AI47714.

Respectfully submitted,

Signature

Janell Cleveland

Date

May 24, 2004

Typed or Printed Name:

Janell T. Cleveland

Registration No.

53,848

CERTIFICATE OF EXPRESS MAILING UNDER 37 C.F.R. § 1.10

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Janell Cleveland

Janell T. Cleveland

May 24, 2004

Date



ATTORNEY DOCKET NO. 21085.0123U1
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)
Kappes et al.)
Application No. Unassigned)
Filing Date: Concurrently) Confirmation No. Unassigned)
For: **METHODS AND COMPOSITIONS FOR**)
IDENTIFYING COMPOUNDS THAT INHIBIT)
HIV-1 SUBUNIT-SPECIFIC REVERSE)
TRANSCRIPTASE)

AUTHORIZATION TO TREAT REPLY REQUIRING EXTENSION OF TIME
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Mail Stop PROVISIONAL PATENT APPLICATION
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NEEDLE & ROSENBERG, P.C.
Customer Number 23859

Sir:

Pursuant to 37 C.F.R. § 1.136(a)(3), the Commissioner is hereby requested and authorized to treat any concurrent or future reply in the above-identified application, requiring a petition for an extension of time for its timely submission, as incorporating a petition for extension of time for the appropriate length of time.

**ATTORNEY DOCKET NO. 21085.0123U1
PATENT**

The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 14-0629.

Respectfully submitted,

NEEDLE & ROSENBERG, P.C.

Janell Cleveland
Janell T. Cleveland
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Customer No. 23859

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EXPRESS MAIL NO. EL 980236563
ATTORNEY DOCKET NO. 21085.0123U1
PROVISIONAL PATENT

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**PROVISIONAL
APPLICATION
FOR
UNITED STATES LETTERS PATENT
FOR**

15

**METHODS AND COMPOSITIONS FOR IDENTIFYING COMPOUNDS
THAT INHIBIT HIV-1 SUBUNIT-SPECIFIC REVERSE TRANSCRIPTASE
BY**

20

John C. Kappes, residing at 5284 Birdsong Rd., Birmingham AL 35242; Alok
25 Mulky, residing at 1850 Arboretum Circle, Apt D Birmingham, AL 35216; and
Xiaoyun Wu, residing at 4217 Heritage Oak Circle, Birmingham, AL 35242.

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ATTORNEY DOCKET NO. 21085.0123U1

**METHODS AND COMPOSITIONS FOR IDENTIFYING COMPOUNDS
THAT INHIBIT HIV-1 SUBUNIT-SPECIFIC REVERSE TRANSCRIPTASE**

This invention was funded by the National Institutes of Health, Grant No.
5 AI47714. Therefore, the United States Government may have certain rights in this
invention.

BACKGROUND OF THE INVENTION

10 **BACKGROUND ART**

The HIV type 1 (HIV-1) reverse transcriptase (RT) is required for the
conversion of genomic RNA into double-stranded proviral DNA, catalyzed by the
RNA- and DNA-dependent polymerase and ribonuclease H activities of the enzyme.
HIV-1 RT is an asymmetric dimer formed by the association of p66 and p51
15 polypeptides, which are cleaved from a large Pr¹⁶⁰GagPol precursor by the viral
protease during virion assembly. p51 contains identical N-terminal sequences as p66,
but lacks the C-terminal ribonuclease H (RNase H) domain (di Marzo et al. Science
231, 1289-1291, 1986). The structure of HIV-1 RT has been elucidated by x-ray
crystallography in a variety of configurations, including unliganded (Rodgers et al.
20 Proc. Natl. Acad. Sci. USA 92, 1222-1226, 1995), complexed to nonnucleoside RT
inhibitors (Ren, et al. Nat. Struct. Biol. 2, 293-302, 1995), or complexed with double-
stranded DNA either with (Huanget al. Science 282, 1669-1675, 1998) or without
deoxynucleotide triphosphate (Jacobo-Molina et al. Proc. Natl. Acad. Sci. USA 90,
6320-6324, 1993; Kohlstaedt et al. Science 256, 1783-1790, 1992). Such analyses
25 have shown that p66 can be divided structurally into the polymerase and RNase H
domains, with the polymerase domain further divided into the fingers, palm, thumb
and connections subdomains. Although p51 has the same polymerase domains as p66,
the relative orientations of these individual domains differ markedly, resulting in p51
assuming a closed structure.

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The RT heterodimer represents the biologically relevant form of the enzyme; the monomeric subunits have only low catalytic activity (Restle, et al. *J. Biol. Chem.* 265, 8986-8988, 1990). Structural analysis reveals three major contacts between p66 and p51, with most of the interaction surfaces being largely hydrophobic (Becerra et al
5 *Biochemistry* 30, 11707-11719, 1991; Wang et al. *Proc. Natl. Acad. Sci. USA* 91, 7242-7246, 1994). The three contacts comprise an extensive dimer interface that includes the fingers subdomain of p51 with the palm of p66, the connection subdomains of both subunits, and the thumb subdomain of p51 with the RNase H domain of p66.

10 Several single amino acid substitutions in HIV-1 RT have been shown to inhibit heterodimer association (Ghosh et al. *Biochemistry* 35, 8553-8562 1996; Wohrl et al. *J. Biol. Chem.* 272, 17581-17587, 1997; Goel et al. *Biochemistry* 32, 13012-13018, 1993). These include the mutations L234A, G231A and W229A, all located in the primer grip region of the p66 subunit, and L289K in the thumb
15 subdomain. Remarkably, these mutations are not located at the dimer interface and probably mediate their effects indirectly through conformational changes in the p66 subunit.

Several biochemical assays have been used previously to specifically measure RT dimerization. Some are based on the physical separation of monomers and dimers
20 as determined by analytical ultracentrifugation and gel filtration. Other assays include intrinsic tryptophan fluorescence (Divita et al. *FEBS Lett.* 324, 153-158, 1993), chemical crosslinking (Debyser et al *Protein Sci.* 5, 278-286, 1996), the use of affinity tags (Jacques et al *J. Biol. Chem.* 269, 1388-1393, 1994) and polymerase activity itself. Although these methods detect dimerization, they either lack specificity or are
25 not easy to perform. Moreover, these assays do not facilitate the rapid genetic analysis of protein-protein interactions under physiological conditions nor are they suitable for high throughput screening for RT dimerization inhibitors.

Understanding the role of the individual RT subunits in RNA- and DNA-

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dependent DNA synthesis has been the focus of several studies. These used *in vitro* biochemical methods to analyze the enzymatic activity of purified recombinant HIV-1 RT heterodimers wherein either the p51 or p66 subunit was selectively mutated (Boyer et al., 1994; Hostomsky et al., 1992; Le Grice et al., 1991). What is needed in the art are *in vivo* methods and compositions for identifying compounds that inhibit HIV-1 subunit-specific reverse transcriptase.

SUMMARY OF THE INVENTION

In accordance with the purpose(s) of this invention, as embodied and broadly described herein, this invention, in one aspect, relates to a cell comprising a vector wherein the vector expresses a fusion protein comprising a p51 subunit and Vpr, and a reverse transcriptase deficient proviral DNA.

In another aspect, the invention relates to a method of screening for a compound that inhibits viral reverse transcriptase.

This invention also relates to a method of screening for a compound that inhibits or enhances dimerization of a p66 subunit polypeptide of reverse transcriptase and a p51 subunit polypeptide of reverse transcriptase.

In another aspect, this invention relates to a method of making a pharmaceutical composition and compounds identified by the methods described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate embodiments of the invention and together with the description, serve to explain the principles of the invention.

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Figure 1 shows construction of the *vpr-p51/p66* and FN expression plasmids.

(A) Illustration of the *vpr-p51/p66* expression plasmid. The *vpr-p51/p66* expression plasmid was constructed to allow independent expression and subunit-specific analysis of p51 and p66. The *vpr* and p51 coding sequences were fused in-frame, while preserving the N-terminal protease cleavage (PC) site of RT by including 33 base-pairs of contiguous PR sequence 5' of RT. A translational stop codon (TAA) was introduced to terminate RT expression at amino acids 440, which represents the full-length p51 subunit. *vpr-p51* was succeeded by an internal ribosome entry site (IRES). The p66 coding region was inserted 3' of the IRES and was modified to encode Met-Gly on the N-terminus. The *vpr-p51/p66* expression plasmid was used to construct various p51/p66 mutants. Unless otherwise indicated, this was accomplished by inserting p51 or p66 DNA fragments at the *BglII-MluI* or *XmaI-XhoI* sites, respectively. (B) Illustration of the FN proviral construct. This proviral construct was made from the wild-type pSG3 plasmid using a previously described strategy (Dubay et al., 1992). The clone contains a 110 amino acid deletion (nucleotides 3374-3704) in the RT reading frame. Most of the RNase H domain and 13 amino acids of the carboxyl end of the polymerase domain were removed, leaving the IN coding region in-frame.

Figure 2 shows a model for *trans* expression and packaging of heterodimeric RT. Cells are cotransfected with HIV-1 and the *vpr-p51/p66* expression plasmids. Vpr-p51 incorporates p66 through interaction and stable association of the two subunits (Vpr-p51 and p66) within the cellular cytoplasm. Specific interaction between Vpr and Pr55^{Gag} leads to the incorporation of the Vpr-p51/p66 complex into progeny virions. Subsequent cleavage by the viral PR generates wild-type RT heterodimer (p51/p66).

Figure 3 shows virion incorporation and proteolytic processing of *trans*-heterodimeric RT. The FN proviral DNA was transfected alone or cotransfected with

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either the *vpr-p66*, *vpr-p51/p66*, or *vprΔp51/p66* expression plasmids. Included as controls were the wild-type SG3 and the RT-IN minus SG3^{S-RT} proviruses. The transfection-derived virions were concentrated by ultracentrifugation, lysed and analyzed by immunoblotting using (A) anti-RT (α-RT), (B) anti-p66 (α-p66) or (C) anti-Gag (α-CA) antibodies.

Figure 4 shows complementation of the M7 provirus eliminates non-Vpr-p51-mediated p66 incorporation. (A) Construction of the M7 proviral plasmid. The M7 construct was derived from the S-RT construct (Wu et al., 1997), which contains a TAA stop codon at the first amino acid positions of RT and IN. In addition to these mutations, M7 has a -1 frameshift at amino acid position 14 of RT, three stop codons, 441 (TAA), 444 (TGA) and 447 (TAG), in the RNase H domain and a RNase H catalytic site mutation at 443 (D443N). (B to D) Analysis of virion incorporation and proteolytic processing of Vpr-p51/p66. The M7 proviral DNA was transfected alone or together with the *vpr-IN* expression plasmid and either the *vpr-p66*, *vpr-p51/p66*, or *vprΔp51/p66* expression plasmids. The wild-type SG3 was included as a control. Transfection-derived virions were concentrated by ultracentrifugation, lysed and analyzed by immunoblotting using (B) anti-RT (α-RT), (C) anti-p66 (α-p66) or (D) anti-Gag (α-CA) antibodies.

Figure 5 shows infectivity of *trans*-heterodimeric complemented virions. Viruses were derived by transfection of 293T cells as described in Fig. 4 and analyzed for HIV-1 p24-ag concentration. Virus infectivity was analyzed using the TZM-bl reporter cell line as described in Example 1. Infectivity is expressed as a percentage of the wild-type virus control. The results of three independent experiments are shown.

Figure 6 shows subunit-specific analysis of the YMDD motif. The wild-type, control and mutated *vpr-p51*/expression plasmids, respectively, were cotransfected

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into 293T cells with the M7 and vpr-IN DNAs. Transfection derived viruses were analyzed for HIV-1 p24-ag concentration. (A) Analysis of infectivity. Infectivity was analyzed from three independent experiments. (B & C) Analysis of viral DNA synthesis. The DNA products of reverse transcription were analyzed as described in
5 Example 1. (B) Early (R-U5) and (C) late (R-gag) products of reverse transcription were amplified from each DNA extract by PCR, resolved on 1.5% agarose gels and stained with ethidium bromide. To approximate the relative amount of each of the amplified DNA products, 10-fold serial dilutions of pSG3 DNA (ranging from 10^1 to 10^5 copies) were prepared and analyzed in parallel. Distilled water (dw) was included
10 as a negative control.

Figure 7 shows interactions of the p51 YMDD (SEQ ID NO: 8) motif (yellow) of HIV-1 reverse transcriptase at the junction of the p51 palm (cyan and yellow), p51 connection (white) and p51 fingers (green) subdomains in the structure of the RT/DNA/dNTP complex (pdb code 1RTD). The Trp-rich region is shown at
15 the interface of the p51 (white) and p66 (red) subunits and proximal to the DNA-binding cleft (red).

Figure 8 shows infectivity for Trp motif mutants. Figure 8A: Lane 1: *trans*-Vprp51/p66 wild-type virus (15-20% of wild-type HIV-1). Normalized to 100%. Lane 2: Background control. Does not express p51 in the *vpr*-p51 reading-frame. Thus, it
20 does not incorporate p66 via Vpr-p51 and there is no active RT in the virion (other than minimal amounts of p66 that could get non-specifically incorporated). Lane 3-9: Mutants in the tryptophan-repeat motif (Trp-motif) of RT. This motif is found the connection subdomain of RT and is unique in having 6 Trp residues. These residues form a hydrophobic cluster of 12 tryptophans spanning the dimerization interface
25 between the RT subunits (p51 and p66). Figure 8B: Lane 1: *trans*-Vpr-p51/p66 wild-type virus Lane 2: Background control. Does not express p51 in the *vpr*-p51 reading-frame. Thus, it does not incorporate p66 via Vpr-p51 and there is no active RT in the

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virion (other than minimal amounts of p66 that could get non-specifically incorporated). Lane 3-9: Mutants in the tryptophan-repeat motif (Trp-motif) of RT. The results of the RT assay are different in that clones like p51W401/p66 (lane 4) have background levels of activity (Vpr-Dp51/p66) in this biochemical assay
5 (Example 2) although this mutant is wild-type on infectivity analysis.

Figure 9 shows infectivity for p51W401-p66W410 dimer interface. Figure 9B: Lane 1: *trans*-Vpr-p51/p66 wild-type virus (15-20% of wild-type HIV-1). Normalized to 100%. Lane 2: Background control. Does not express p51 in the *vpr*-p51 reading-frame. Thus, it does not incorporate p66 via Vpr-p51 and there is no
10 active RT in the virion (other than minimal amounts of p66 that could get non-specifically incorporated). The residues p51W401 and p66W410 are at the interface between p51 and p66 within interacting distance (~3Å) based on crystal structure. These residues were mutated both individually (lanes 3-6) and together (lanes 7-9). The single mutants do not have much effect on infectivity, while the double mutants
15 have a greater effect. The p51/p66L234A (lane 10) and p51W401A/p66 (lane 11) are well-established dimerization defective mutants identified by biochemical and yeast-2-hybrid assay recognized in the field to be defective in RT assays (biochemical). It is quite clear from these controls that biochemical data do not accurately reflect what occurs in the virion. Figure 9B: Lane 1: *trans*-Vpr-p51/p66 wild-type virus (15-20%
20 of wild-type HIV-1). Lane 2: Background control. Does not express p51 in the *vpr*-p51 reading-frame. Thus, it does not incorporate p66 via Vpr-p51 and there is no active RT in the virion (other than minimal amounts of p66 that could get non-specifically incorporated). The residues p51W401 and p66W410 are at the interface between p51 and p66 within interacting distance (~3Å) based on crystal structure.
25 These residues were both mutated individually (lanes 3-6) and together (lanes 7-9). The single mutants to reduce RT (biochemical) activity to background levels (Vpr-Dp51/p66), while the double mutants have a greater effect and reduce the RT activity to negative control levels. The p51/p66L234A (lane 10) and p51W401A/p66 (lane

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11), well-established dimerization defective mutants identified by biochemical and yeast-2-hybrid assay recognized in the field to be defective in RT assays (biochemical, Example 2) are also defective in our RT assay at negative control levels. It is quite clear from this data that biochemical data does not accurately reflect what happens in the virion.

DETAILED DESCRIPTION OF THE INVENTION

The present invention may be understood more readily by reference to the following detailed description of preferred embodiments of the invention and the Examples included therein and to the Figures and their previous and following description.

DEFINITIONS

As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a small molecule" includes mixtures of one or more small molecules, and the like.

Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, this includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint.

The terms "higher," "increases," "elevates," "enhances" or "elevation" refer to increases above basal levels, or as compared to a control. The terms "low," "lower," "reduces," "inhibits" "decreases" or "reduction" refer to decreases below

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basal levels, or as compared to a control. For example, basal levels are normal *in vivo* levels prior to, or in the absence of, the vector, or addition of an agent such as or another small molecule or ligand.

5 The term "test compound" is defined as any compound to be tested for its ability to interact with a selected cell.

The terms "control levels" or "control cells" are defined as the standard by which a change is measured, for example, the controls are not subjected to variables, but are instead subjected to a defined set of parameters in the absence of variables, or the controls are based on pre- or post-variable levels.

10 The terms "polypeptide," "peptide," and "protein" are used interchangeably throughout and are defined as sequences containing amino acids.

GENERAL

The biologically relevant, catalytically active form of human immunodeficiency virus type-1 (HIV-1) reverse transcriptase (RT) is a heterodimer consisting of a 51 kDa subunit and a 66 kDa subunit. Since p51 and p66 are derived from the same coding region, subunit-specific structure/function studies of RT have not been possible *in vivo*. RT has both DNA polymerase and RNase H activities that are required to convert the single-stranded RNA viral genome into double-stranded DNA upon entry of the virus into host cells.

20 RT is translated and assembled into virions as part of a larger Gag-Pol polyprotein precursor (Pr160^{Gag-Pol}). Proteolytic processing of Pr160^{Gag-Pol} by the *pol*-encoded protease (PR) generates the mature heterodimeric form (p51/p66) of the RT enzyme (Freed and Martin, 2001; Telesnitsky and Goff, 1997). The N-terminal 440 amino acids of p51 and p66 are collinear. The p66 subunit contains the DNA
25 polymerase and RNase H domains, while the p51 subunit lacks the RNase H domain (Hizi et al., 1988; Larder et al., 1987a; Prasad and Goff, 1989). Elucidation of the

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HIV-1 RT structure has shown that the polymerase domain of p51 and p66 can be further divided into the fingers, palm, thumb, and connection subdomains (Kohlstaedt et al., 1992). Although both p51 and p66 contain each of these subdomains, their relative arrangements differ markedly between the two subunits. Since these subunits
5 are derived from the same coding region, a mutation in the polymerase coding region generates a heterodimer that contains a mutation in each subunit. However, as their structures are different in the heterodimer, the effect of these mutations on RT subunit structure/function is not equivalent (Arnold et al., 1992; Kohlstaedt et al., 1992). Thus, the heterodimeric nature of RT has previously had limited detailed molecular
10 genetic analyses of the p51 and p66 subunit function

Viral and foreign proteins can be incorporated into virions by exploiting viral accessory proteins, such as HIV/SIV proteins Vpr or Vpx, as targeting vehicles. By expressing the desired protein in *trans* as a fusion with Vpr or Vpx, its incorporation is brought about through an interaction between Vpr/Vpx and the p6 domain of the
15 cognate Gag precursor polyprotein (Lu et al., 1993; Paxton et al., 1993; Wu et al., 1994). Using this approach, it has been shown that HIV-1 RT and IN functions can be provided when expressed in *trans* as Vpr fusion proteins, independently of Pr160^{Gag-Pol} (Liu et al., 1997; Wu et al., 1999; Wu et al., 1997).

Herein described is a *trans*-complementation approach that enables the
20 function of the individual RT subunits to be analyzed in the context of an infectious virus. For example, by cotransfecting cells with RT-defective proviral DNA and an LTR-vpr-p51-IRES-p66 expression cassette, it was demonstrated that Vpr-p51 interacts with p66 and mediates virion incorporation of a Vpr-p51/p66 heterodimeric complex. The p51 subunit was expressed as a Vpr-p51 fusion protein that incorporates
25 into HIV-1 virions through an interaction between Vpr and the Gag precursor polyprotein. When coexpressed, p66 is specifically and selectively packaged as a Vpr-p51/p66 complex. Processing by the viral protease liberates Vpr and generates

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functional heterodimeric RT (p51/p66) that supports HIV-1 reverse transcription and virus infection (Example 1).

5 This approach was used to demonstrate that the YMDD aspartates of p66 are both required and sufficient for RT polymerase function, and that the p51 YMDD aspartates play a structural role that is required for viral cDNA synthesis in infected cells. By mutating D185 and D186 of either p51 or p66, the role of these residues, for the first time, in the context of an infectious virus, were studied. The results corroborate earlier findings that the aspartates of p66 (YMDD) are required and sufficient for polymerase function of the RT heterodimer. Decreased viral DNA
10 synthesis and infectivity was observed with certain p51 aspartate mutations (YMDD), indicating that both the occupancy and charge of these residues are important for RT function *in vivo*. These findings demonstrate detailed molecular genetic and biologic analyses of the RT subunits *in vivo*.

COMPOSITIONS

15 Disclosed are the components to be used to prepare the disclosed compositions as well as the compositions themselves to be used within the methods disclosed herein. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and
20 permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular plasmid is disclosed and discussed and a number of modifications that can be made to a number of molecules included in the plasmid are discussed, specifically contemplated is each and every combination and permutation of those molecules and the modifications that are
25 possible unless specifically indicated to the contrary. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited

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each is individually and collectively contemplated meaning combinations, A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are considered disclosed. Likewise, any subset or combination of these is also disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E would be considered disclosed. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods.

Disclosed herein are plasmids comprising a fusion protein comprising a p51-containing DNA fragment fused in frame and a viral accessory protein, such as *vpr*. By expressing the desired protein in *trans* as a fusion with Vpr, for example, its incorporation is brought about through an interaction between Vpr and the p6 domain of the cognate Gag precursor polyprotein. The Vpr-p51 fusion includes the natural PR-RT cleavage site (PC), allowing processing by the viral protease and liberation of Vpr (Wu et al., 1997). Also disclosed herein is an expression cassette comprising LTR-vpr-p51-IRES-p66, wherein the nucleic acid comprises SEQ ID NO: 1.

Also disclosed herein are vectors comprising a p66 subunit, a p51 subunit, and Vpr, wherein Vpr and p51 are expressed as a fusion protein. The p66 and p51 subunits can be expressed on the same, or on different, mRNAs.

Optionally, an internal ribosome entry site (IRES) can be placed downstream of *vpr-p51*, followed by the p66 coding sequence. IRES are *cis*-acting RNA sequences able to mediate internal entry of a sequence on some eukaryotic and viral messenger RNAs upstream of a translation initiation codon. Examples of useful IRES can be found at <http://ifr31w3.toulouse.inserm.fr/IRESdatabase>, herein incorporated by reference in its entirety for the disclosure of various IRES.

Transcription of *vpr-p51/p66* can then be placed under the control of a long terminal repeat (LTR), for example. LTRs are responsible for integration of the

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sequence into the host genome, initiation and enhancement of retroviral transcription, as well as transcriptional termination, and modulation of retroviral replication levels. Examples of LTRs useful with the plasmids described herein include SIV-LTR, HIV-1 LTR, and HIV-2 LTR, for example.

5 The plasmid can be incorporated into proviral clones that contain a deletion in RT. For example, the proviral clone pSG3^{FN} (Figure 1B) was used to study incorporation of the heterodimeric *trans*-RT into virions when coexpressed with the *vpr-p51/p66* expression plasmid (Example 1). The FN clone was selected for this purpose since it contains a deletion in RT that includes most of the RNase H region and extends 13 amino acids into the carboxyl-terminus of the p51 domain, however, any proviral clone can be used for this purpose. This created a defective RT, while the *pol* reading frame, including IN, remained open.

10 An expression plasmid including IN, such as *vpr-IN*, can also be included in conjunction with the plasmid disclosed herein. The M7 clone (pSG3^{FN}) does not express the IN protein, and integration of the nascent viral cDNA is required to detect infection. Moreover, IN is also required for efficient initiation of reverse transcription (Wu et al., 1999).

15 Effective *trans*-complementation requires expression of the two subunits (Vpr-p51 and p66), dimerization, and stable association of the p51 (Vpr-p51) and p66 subunits within the cytosol of the cell, specific interaction of Vpr with Pr55^{Gag}, incorporation of the Vpr-p51/p66 heterodimeric complex into virions, proteolytic cleavage to liberate Vpr from p51/p66, and proper interaction of RT with the template-primer.

20 Effective *trans*-complementation requires expression of the two subunits (Vpr-p51 and p66), dimerization, and stable association of the p51 (Vpr-p51) and p66 subunits within the cytosol of the cell, specific interaction of Vpr with Pr55^{Gag}, incorporation of the Vpr-p51/p66 heterodimeric complex into virions, proteolytic cleavage to liberate Vpr from p51/p66, and proper interaction of RT with the template-primer.

25 Also disclosed herein are cells comprising: (i) a vector comprising a p66 subunit, a p51 subunit, and Vpr, wherein Vpr and p51 are expressed as a fusion protein; (ii) and a reverse transcriptase deficient proviral DNA. One example of a cell that can be used is the 293T cell.

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Also disclosed are cell lines stably transformed with the plasmid described herein. For example, the cell line can comprise an exogenous nucleic acid, the nucleic acid comprising vpr-p51/66. The cell line can express viral nucleic acids as well, and can be induced to express viral nucleic acids by contacting the cell with a stimulus.

- 5 An example of such a stimulus includes, but is not limited to, tetracycline. Also disclosed are transgenic animals expressing vpr-p51/66.

Homology/identity.

- It is understood that one way to define any known variants and derivatives or those that might arise, of the disclosed genes and proteins herein is through defining the variants and derivatives in terms of homology to specific known sequences. For example SEQ ID NO: 1 sets forth a particular nucleic acid sequence encoding an expression protein and SEQ ID NO 2 sets forth a particular sequence of the protein encoded by *vpr*. Specifically disclosed are variants of these and other genes and proteins herein disclosed which have at least, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 percent homology to the stated sequence. Those of skill in the art readily understand how to determine the homology of two proteins or nucleic acids, such as genes. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level. Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman Adv. Appl. Math. 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, J. MoL Biol. 48: 443 (1970), by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. U.S.A. 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.
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The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. *Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are herein incorporated by reference for at least material related to
5 nucleic acid alignment.

Nucleic acids

There are a variety of molecules disclosed herein that are nucleic acid based, including for example the nucleic acids of the plasmid disclosed herein, as well as those that encode the proteins disclosed herein, as well as various functional nucleic
10 acids. The disclosed nucleic acids are made up of for example, nucleotides, nucleotide analogs, or nucleotide substitutes. Non-limiting examples of these and other molecules are discussed herein. It is understood that for example, when a vector is expressed in a cell, that the expressed mRNA will typically be made up of A, C, G, and U. Likewise, it is understood that if, for example, an antisense molecule is
15 introduced into a cell or cell environment through for example exogenous delivery, it is advantageous that the antisense molecule be made up of nucleotide analogs that reduce the degradation of the antisense molecule in the cellular environment.

Nucleotides and related molecules

A nucleotide is a molecule that contains a base moiety, a sugar moiety and a
20 phosphate moiety. Nucleotides can be linked together through their phosphate moieties and sugar moieties creating an internucleoside linkage. The base moiety of a nucleotide can be adenin-9-yl (A), cytosin-1-yl (C), guanin-9-yl (G), uracil-1-yl (U), and thymine-1-yl (T). The sugar moiety of a nucleotide is a ribose or a deoxyribose. The phosphate moiety of a nucleotide is pentavalent phosphate. An non-limiting
25 example of a nucleotide would be 3'-AMP (3'-adenosine monophosphate) or 5'-GMP (5'-guanosine monophosphate).

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A nucleotide analog is a nucleotide which contains some type of modification to either the base, sugar, or phosphate moieties. Modifications to nucleotides are well known in the art and would include for example, 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, and 2-aminoadenine as well as
5 modifications at the sugar or phosphate moieties.

Nucleotide substitutes are molecules having similar functional properties to nucleotides, but which do not contain a phosphate moiety, such as peptide nucleic acid (PNA). Nucleotide substitutes are molecules that will recognize nucleic acids in a Watson-Crick or Hoogsteen manner, but which are linked together through a moiety
10 other than a phosphate moiety. Nucleotide substitutes are able to conform to a double helix type structure when interacting with the appropriate target nucleic acid.

It is also possible to link other types of molecules (conjugates) to nucleotides or nucleotide analogs to enhance for example, cellular uptake. Conjugates can be chemically linked to the nucleotide or nucleotide analogs. Such conjugates include
15 but are not limited to lipid moieties such as a cholesterol moiety. (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989,86, 6553-6556),

A Watson-Crick interaction is at least one interaction with the Watson-Crick face of a nucleotide, nucleotide analog, or nucleotide substitute. The Watson-Crick face of a nucleotide, nucleotide analog, or nucleotide substitute includes the C2, N1,
20 and C6 positions of a purine based nucleotide, nucleotide analog, or nucleotide substitute and the C2, N3, C4 positions of a pyrimidine based nucleotide, nucleotide analog, or nucleotide substitute.

A Hoogsteen interaction is the interaction that takes place on the Hoogsteen face of a nucleotide or nucleotide analog, which is exposed in the major groove of
25 duplex DNA. The Hoogsteen face includes the N7 position and reactive groups (NH₂ or O) at the C6 position of purine nucleotides.

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Sequences

There are a variety of sequences related to, for example, the plasmid described herein, as well as any other protein disclosed herein that are disclosed on Genbank, and these sequences and others are herein incorporated by reference in their entireties
5 as well as for individual subsequences contained therein.

A variety of sequences are provided herein and these and others can be found in Genbank, at www.pubmed.gov. Those of skill in the art understand how to resolve sequence discrepancies and differences and to adjust the compositions and methods relating to a particular sequence to other related sequences.

10 *Peptides*

As discussed herein there are numerous variants of the vectors disclosed herein that are known and herein contemplated. In addition to the known functional variants there are derivatives of the proteins disclosed herein, such as Vpr, p51, or p66, which also function in the disclosed methods and compositions. Protein variants and
15 derivatives are well understood to those of skill in the art and in can involve amino acid sequence modifications. For example, amino acid sequence modifications typically fall into one or more of three classes: substitutional, insertional or deletional variants. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Insertions
20 ordinarily will be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of one to four residues. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. Typically, no more than about from 2 to 6 residues are deleted at any one site within the protein molecule. These variants ordinarily are prepared by site specific mutagenesis of
25 nucleotides in the DNA encoding the protein, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. Techniques for making substitution mutations at predetermined sites in DNA having a known

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sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis.

Specifically, mutations can occur in p51, p66, Vpr, IRES, or any of the nucleic acids encoding these peptides. Mutations can also occur in the *env* gene of HIV, for example, which can optionally affect the infectivity of the virus. These mutations can be deletions, substitutions, or insertion mutations. The mutations can occur in RT and/or in IN. The mutations can also be point mutations.

Amino acid substitutions are typically of single residues, but can occur at a number of different locations at once; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. The mutations must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Tables 1 and 2 and are referred to as conservative substitutions.

TABLE 1: Amino Acid Abbreviations

Amino Acid	Abbreviations
alanine	AlaA
allosoleucine	Alle
arginine	ArgR
asparagine	AsnN
aspartic acid	AspD

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Amino Acid	Abbreviations
cysteine	CysC
glutamic acid	GluE
glutamine	GlnK
glycine	GlyG
histidine	HisH
isoleucine	IleI
leucine	LeuL
lysine	LysK
phenylalanine	PheF
proline	ProP
pyroglutamic acid	Glu
Serine	SerS
Threonine	ThrT
Tyrosine	TyrY
Tryptophan	TrpW
Valine	ValV

TABLE 2:Amino Acid Substitutions
Original Residue Exemplary Conservative Substitutions, others are known in the art.
Ala-ser
Arg-lys, gln
Asn-gln; his
Asp-glu
Cys-ser

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Gln-asn, lys
Glu-asp
Gly-pro
His-asn;gln
Ile-leu; val
Leu-ile; val
Lys-arg; gln;
Met-Leu; ile
Phe-met; leu; tyr
Ser-thr
Thr-ser
Trp-tyr
Tyr-trp; phe
Val-ile; leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the protein properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g.,

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phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine, in this case, (e) by increasing the number of sites for sulfation and/or glycosylation.

For example, the replacement of one amino acid residue with another that is biologically and/or chemically similar is known to those skilled in the art as a conservative substitution. For example, a conservative substitution would be replacing one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as, for example, Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such conservatively substituted variations of each explicitly disclosed sequence are included within the mosaic polypeptides provided herein.

Substitutional or deletional mutagenesis can be employed to insert sites for N-glycosylation (Asn-X-Thr/Ser) or O-glycosylation (Ser or Thr). Deletions of cysteine or other labile residues also may be desirable. Deletions or substitutions of potential proteolysis sites, e.g. Arg, is accomplished for example by deleting one of the basic residues or substituting one by glutaminy or histidyl residues.

Certain post-translational derivatizations are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminy and asparaginy residues are frequently post-translationally deamidated to the corresponding glutamyl and asparyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the o-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecular Properties*, W. H. Freeman & Co., San Francisco pp 79-86 [1983]), acetylation of the N-terminal amine and, in some instances, amidation of the C-terminal carboxyl.

It is understood that one way to define the variants and derivatives of the disclosed proteins herein is through defining the variants and derivatives in terms of

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homology/identity to specific known sequences. For example, SEQ ID NO: 1 sets forth a particular nucleic acid sequence of a vector described herein, which encodes the Vpr and p51 subunits; and SEQ ID NO: 2 sets forth a particular sequence of a Vpr protein. Specifically disclosed are variants of these and other proteins herein
5 disclosed which have at least, 70% or 75% or 80% or 85% or 90% or 95% homology to the stated sequence. Those of skill in the art readily understand how to determine the homology of two proteins. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

Another way of calculating homology can be performed by published
10 algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444 (1988), by computerized implementations of these
15 algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. *Science* 244:48-52, 1989, Jaeger et al. *Proc.*
20 *Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment.

It is understood that the description of conservative mutations and homology can be combined together in any combination, such as embodiments that have at least
25 70% homology to a particular sequence wherein the variants are conservative mutations.

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As this specification discusses various proteins and protein sequences it is understood that the nucleic acids that can encode those protein sequences are also disclosed. This would include all degenerate sequences related to a specific protein sequence, i.e. all nucleic acids having a sequence that encodes one particular protein sequence as well as all nucleic acids, including degenerate nucleic acids, encoding the disclosed variants and derivatives of the protein sequences. Thus, while each particular nucleic acid sequence may not be written out herein, it is understood that each and every sequence is in fact disclosed and described herein through the disclosed protein sequence.

It is understood that there are numerous amino acid and peptide analogs which can be incorporated into the disclosed compositions. For example, there are numerous D amino acids or amino acids which have a different functional substituent than the amino acids shown in Table 1 and Table 2. The opposite stereo isomers of naturally occurring peptides are disclosed, as well as the stereo isomers of peptide analogs. These amino acids can readily be incorporated into polypeptide chains by charging tRNA molecules with the amino acid of choice and engineering genetic constructs that utilize, for example, amber codons, to insert the analog amino acid into a peptide chain in a site specific way (Thorson et al., Methods in Molec. Biol. 77:43-73 (1991), Zoller, Current Opinion in Biotechnology, 3:348-354 (1992); Ibba, Biotechnology & Genetic Engineering Reviews 13:197-216 (1995), Cahill et al., TIBS, 14(10):400-403 (1989); Benner, TIB Tech, 12:158-163 (1994); Ibba and Hennecke, Bio/technology, 12:678-682 (1994) all of which are herein incorporated by reference at least for material related to amino acid analogs).

Molecules can be produced that resemble peptides, but which are not connected via a natural peptide linkage. For example, linkages for amino acids or amino acid analogs can include $\text{CH}_2\text{NH--}$, $\text{--CH}_2\text{S--}$, $\text{--CH}_2\text{--CH}_2\text{--}$, --CH=CH-- (cis and trans), $\text{--COCH}_2\text{--}$, $\text{--CH(OH)CH}_2\text{--}$, and $\text{--CHH}_2\text{SO--}$ (These and others can be

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found in Spatola, A. F. in Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3, Peptide Backbone Modifications (general review); Morley, Trends Pharm Sci (1980) pp. 463-468; Hudson, D. et al., Int J Pept
5 Prot Res 14:177-185 (1979) (--CH₂NH--, CH₂CH₂--); Spatola et al. Life Sci 38:1243-1249 (1986) (--CH H₂--S); Hann J. Chem. Soc Perkin Trans. I 307-314 (1982) (--CH--CH--, cis and trans); Almquist et al. J. Med. Chem. 23:1392-1398 (1980) (--COCH₂--); Jennings-White et al. Tetrahedron Lett 23:2533 (1982) (--COCH₂--); Szelke et al. European Appln, EP 45665 CA (1982): 97:39405 (1982) (--CH(OH)CH₂--); Holladay
10 et al. Tetrahedron. Lett 24:4401-4404 (1983) (--C(OH)CH₂--); and Hruby Life Sci 31:189-199 (1982) (--CH₂--S--); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is --CH₂NH--. It is understood that peptide analogs can have more than one atom between the bond atoms, such as β -alanine, γ -aminobutyric acid, and the like.

15 Amino acid analogs and analogs and peptide analogs often have enhanced or desirable properties, such as, more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others.

20 D-amino acids can be used to generate more stable peptides, because D amino acids are not recognized by peptidases and such. Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) can be used to generate more stable peptides. Cysteine residues can be used to cyclize or attach two or more peptides together. This can be
25 beneficial to constrain peptides into particular conformations. (Rizo and Gierasch Ann. Rev. Biochem. 61:387 (1992), incorporated herein by reference).

Functional Nucleic Acids

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Functional nucleic acids are nucleic acid molecules that have a specific function, such as binding a target molecule or catalyzing a specific reaction. The compositions and methods described herein can be used with any functional nucleic acid. Functional nucleic acid molecules can be divided into the following categories, which are not meant to be limiting. For example, functional nucleic acids include antisense molecules, aptamers, ribozymes, triplex forming molecules, and external guide sequences. The functional nucleic acid molecules can act as effectors, inhibitors, modulators, and stimulators of a specific activity possessed by a target molecule, or the functional nucleic acid molecules can possess a de novo activity independent of any other molecules.

Functional nucleic acid molecules can interact with any macromolecule, such as DNA, RNA, polypeptides, or carbohydrate chains. Thus, functional nucleic acids can interact with the mRNA of HIV or the genomic DNA of the subject, or they can interact with the polypeptide of the compositions disclosed herein. Often functional nucleic acids are designed to interact with other nucleic acids based on sequence homology between the target molecule and the functional nucleic acid molecule. In other situations, the specific recognition between the functional nucleic acid molecule and the target molecule is not based on sequence homology between the functional nucleic acid molecule and the target molecule, but rather is based on the formation of tertiary structure that allows specific recognition to take place.

Antisense molecules are designed to interact with a target nucleic acid molecule through either canonical or non-canonical base pairing. The interaction of the antisense molecule and the target molecule is designed to promote the destruction of the target molecule through, for example, RNaseH mediated RNA-DNA hybrid degradation. Alternatively the antisense molecule is designed to interrupt a processing function that normally would take place on the target molecule, such as transcription or replication. Antisense molecules can be designed based on the sequence of the

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target molecule. Numerous methods for optimization of antisense efficiency by finding the most accessible regions of the target molecule exist. Exemplary methods would be in vitro selection experiments and DNA modification studies using DMS and DEPC. It is preferred that antisense molecules bind the target molecule with a dissociation constant (k_d) less than or equal to 10^{-6} , 10^{-8} , 10^{-10} , or 10^{-12} . A representative sample of methods and techniques which aid in the design and use of antisense molecules can be found in the following non-limiting list of United States patents: 5,135,917, 5,294,533, 5,627,158, 5,641,754, 5,691,317, 5,780,607, 5,786,138, 5,849,903, 5,856,103, 5,919,772, 5,955,590, 5,990,088, 5,994,320, 5,998,602, 6,005,095, 6,007,995, 6,013,522, 6,017,898, 6,018,042, 6,025,198, 6,033,910, 6,040,296, 6,046,004, 6,046,319, and 6,057,437.

Aptamers are molecules that interact with a target molecule, preferably in a specific way. Typically aptamers are small nucleic acids ranging from 15-50 bases in length that fold into defined secondary and tertiary structures, such as stem-loops or G-quartets. Aptamers can bind small molecules, such as ATP (United States patent 5,631,146) and theophiline (United States patent 5,580,737), as well as large molecules, such as reverse transcriptase (United States patent 5,786,462) and thrombin (United States patent 5,543,293). Aptamers can bind very tightly with k_d s from the target molecule of less than 10^{-12} M. It is preferred that the aptamers bind the target molecule with a k_d less than 10^{-6} , 10^{-8} , 10^{-10} , or 10^{-12} . Aptamers can bind the target molecule with a very high degree of specificity. For example, aptamers have been isolated that have greater than a 10000 fold difference in binding affinities between the target molecule and another molecule that differ at only a single position on the molecule (United States patent 5,543,293). It is preferred that the aptamer have a k_d with the target molecule at least 10, 100, 1000, 10,000, or 100,000 fold lower than the k_d with a background binding molecule. It is preferred when doing the comparison for a polypeptide for example, that the background molecule be a different polypeptide. Representative examples of how to make and use aptamers to

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bind a variety of different target molecules can be found in the following non-limiting list of United States patents: 5,476,766, 5,503,978, 5,631,146, 5,731,424 , 5,780,228, 5,792,613, 5,795,721, 5,846,713, 5,858,660 , 5,861,254, 5,864,026, 5,869,641, 5,958,691, 6,001,988, 6,011,020, 6,013,443, 6,020,130, 6,028,186, 6,030,776, and
5 6,051,698.

Ribozymes are nucleic acid molecules that are capable of catalyzing a chemical reaction, either intramolecularly or intermolecularly. Ribozymes are thus catalytic nucleic acid. It is preferred that the ribozymes catalyze intermolecular reactions. There are a number of different types of ribozymes that catalyze nuclease
10 or nucleic acid polymerase type reactions which are based on ribozymes found in natural systems, such as hammerhead ribozymes, (for example, but not limited to the following United States patents: 5,334,711, 5,436,330, 5,616,466, 5,633,133, 5,646,020, 5,652,094, 5,712,384, 5,770,715, 5,856,463, 5,861,288, 5,891,683, 5,891,684, 5,985,621, 5,989,908, 5,998,193, 5,998,203, WO 9858058 by Ludwig and
15 Sproat, WO 9858057 by Ludwig and Sproat, and WO 9718312 by Ludwig and Sproat) hairpin ribozymes (for example, but not limited to the following United States patents: 5,631,115, 5,646,031, 5,683,902, 5,712,384, 5,856,188, 5,866,701, 5,869,339, and 6,022,962), and tetrahymena ribozymes (for example, but not limited to the following United States patents: 5,595,873 and 5,652,107). There are also a number
20 of ribozymes that are not found in natural systems, but which have been engineered to catalyze specific reactions de novo (for example, but not limited to the following United States patents: 5,580,967, 5,688,670, 5,807,718, and 5,910,408). Preferred ribozymes cleave RNA or DNA substrates, and more preferably cleave RNA substrates. Ribozymes typically cleave nucleic acid substrates through recognition
25 and binding of the target substrate with subsequent cleavage. This recognition is often based mostly on canonical or non-canonical base pair interactions. This property makes ribozymes particularly good candidates for target specific cleavage of nucleic acids because recognition of the target substrate is based on the target substrates

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sequence. Representative examples of how to make and use ribozymes to catalyze a variety of different reactions can be found in the following non-limiting list of United States patents: 5,646,042, 5,693,535, 5,731,295, 5,811,300, 5,837,855, 5,869,253, 5,877,021, 5,877,022, 5,972,699, 5,972,704, 5,989,906, and 6,017,756.

5 Triplex forming functional nucleic acid molecules are molecules that can interact with either double-stranded or single-stranded nucleic acid. When triplex molecules interact with a target region, a structure called a triplex is formed, in which there are three strands of DNA forming a complex dependant on both Watson-Crick and Hoogsteen base-pairing. Triplex molecules are preferred because they can bind
10 target regions with high affinity and specificity. It is preferred that the triplex forming molecules bind the target molecule with a k_d less than 10^{-6} , 10^{-8} , 10^{-10} , or 10^{-12} . Representative examples of how to make and use triplex forming molecules to bind a variety of different target molecules can be found in the following non-limiting list of United States patents: 5,176,996, 5,645,985, 5,650,316, 5,683,874, 5,693,773,
15 5,834,185, 5,869,246, 5,874,566, and 5,962,426.

 External guide sequences (EGSs) are molecules that bind a target nucleic acid molecule forming a complex, and this complex is recognized by RNase P, which cleaves the target molecule. EGSs can be designed to specifically target a RNA molecule of choice. RNase P aids in processing transfer RNA (tRNA) within a cell.
20 Bacterial RNase P can be recruited to cleave virtually any RNA sequence by using an EGS that causes the target RNA:EGS complex to mimic the natural tRNA substrate. (WO 92/03566 by Yale, and Forster and Altman, Science 238:407-409 (1990)).

 Similarly, eukaryotic EGS/RNase P-directed cleavage of RNA can be utilized to cleave desired targets within eukaryotic cells. (Yuan et al., Proc. Natl. Acad. Sci.
25 USA 89:8006-8010 (1992); WO 93/22434 by Yale; WO 95/24489 by Yale; Yuan and Altman, EMBO J 14:159-168 (1995), and Carrara et al., Proc. Natl. Acad. Sci. (USA) 92:2627-2631 (1995)). Representative examples of how to make and use EGS

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molecules to facilitate cleavage of a variety of different target molecules be found in the following non-limiting list of United States patents: 5,168,053, 5,624,824, 5,683,873, 5,728,521, 5,869,248, and 5,877,162.

5 METHODS

Also disclosed herein are methods of screening for a compound that inhibits viral reverse transcriptase comprising: a) contacting a cell comprising (i) a plasmid which expresses a fusion protein comprising a p66 subunit, a p51 subunit, and Vpr, (ii) and a reverse transcriptase deficient proviral DNA with the compound, and b)
10 comparing the level of viral infectivity in the presence of the compound with the level of viral infectivity in the absence of the compound, wherein a decreased level of infectivity in the presence of the compound indicates that the compound inhibits reverse transcriptase.

The compositions and methods described herein can be used to treat
15 retroviruses, and in particular lentiviruses. Lentiviruses share several molecular and pathogenic features that set them apart from other retroviruses. These include virus encoded regulatory proteins to stimulate viral gene expression, synthesis of multiply spliced mRNAs and chronic infection associated with slow development of disease. Lentiviruses include, but are not limited to, HIV-1, HIV-2 and SIV. In the methods
20 described therein, the HIV or SIV particles can be derived by genes expressed in the cell, wherein the genes contain one or more nucleotide mutations. Examples of these specific mutations can be found in Example 1.

The p51 and p66 subunits can be expressed on the same or on different messenger RNAs. Furthermore, expression of Vpr-p51 can incorporate the p66
25 protein into viral particles. The plasmid can also express an internal ribosome entry site (IRES), as described above.

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Also disclosed are methods of screening for a compound that inhibits dimerization of a p66 subunit polypeptide of reverse transcriptase and a p51 subunit polypeptide of reverse transcriptase comprising: a) contacting a cell comprising (i) a plasmid which expresses a fusion protein comprising a p66 subunit, a p51 subunit,
5 and Vpr, (ii) and a reverse transcriptase deficient proviral DNA with the compound, and b) comparing the level of complex formation in the presence of the compound with the level of complex formation in the absence of the compound, a lower level of complex formation indicating that the compound inhibits dimerization of the p66 subunit and a p51 subunit.

10 Also disclosed are methods of screening for a compound that enhances dimerization of a p66 subunit polypeptide of reverse transcriptase and a p51 subunit polypeptide of reverse transcriptase comprising: a) contacting a cell comprising (i) a plasmid which expresses a fusion protein comprising a p66 subunit, a p51 subunit, and Vpr, (ii) and a reverse transcriptase deficient proviral DNA with the compound,
15 and b) comparing the level of complex formation in the presence of the compound with the level of complex formation in the absence of the compound, a lower level of complex formation indicating that the compound enhances dimerization of the p66 subunit and a p51 subunit. Examples of compounds that inhibit reverse transcriptase by enhancing subunit dimerization include, but are not limited to, NNRTI.

20 Also disclosed is a method of inhibiting viral reverse transcriptase comprising contacting (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of the compound identified by the method described above, thereby inhibiting viral reverse transcriptase.

25 Also disclosed is a method of inhibiting dimerization of a p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase, which comprises contacting either (1) the p51 subunit

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polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of the compound identified by the method described above, thereby inhibiting dimerization of the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase.

Also disclosed is a method of enhancing dimerization of a p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase, which comprises contacting either (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of the compound identified by the method described above, thereby enhancing dimerization of the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase.

In the methods described above, HIV-1 reverse transcriptase can be present in a subject, a eukaryotic cell, or a prokaryotic cell, for example.

As used throughout, by a "subject" is meant an individual. Thus, the "subject" can include domesticated animals, such as cats, dogs, etc., livestock (e.g., cattle, horses, pigs, sheep, goats, etc.), laboratory animals (e.g., mouse, rabbit, rat, guinea pig, etc.) and birds. Preferably, the subject is a mammal such as a primate, and, more preferably, a human.

The methods of screening described herein are useful with high throughput screening methods. Screening optionally takes place in multi-well plates. Multi-well plates are standard in the art and come in a variety of sizes and shapes. For example, the multi-well plate can be 24, 48, or 96 well plates. Such screening assays can be automated or further modified for high throughput analysis. For high throughput screening, each well can include numerous test components. If a positive reaction is detected in a well, the screening is repeated with one of the test compounds contained in a single well.

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Optionally, reverse transcriptase containing (vpr-p51/p66) virus particles can be made that either lack Env or contain either autologous or heterologous Env derived by pseudotyping. Wei shows this with autologous Env, (Wei, X., J. M. Decker, H. Liu, Z. Zhang, R. B. Arani, J. M. Kilby, M. S. Saag, X. Wu, G. M. Shaw, and J. C. Kappes. 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob Agents Chemother* 46:1896-905; Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw. 2003. Antibody neutralization and escape by HIV-1. *Nature* 422:307-12; both herein incorporated in their entireties for the teaching of autologous Env); while Wu shows this with the VSV-G Env (Wu, X., J. K. Wakefield, H. Liu, H. Xiao, R. Kralovics, J. T. Prchal, and J. C. Kappes. 2000. Development of a novel trans-lentiviral vector that affords predictable safety. *Mol Ther* 2:47-55, herein incorporated by reference in its entirety for its teaching of VSV-G Env). The Wei citations describe how env minus or env mutant virus can be rendered infectious by providing an envelope glycoprotein in *trans*.

COMPOUNDS AND METHODS OF MAKING

Also disclosed herein are methods of making a pharmaceutical composition which comprises: a) determining whether a compound inhibits reverse transcriptase by the methods described herein; and b) admixing the compound with a pharmaceutically acceptable carrier.

Also disclosed are compounds identified by the methods described herein, as well as compositions comprising the compounds identified by the methods described herein. Such compositions can also comprise a carrier. The compound can be capable of inhibiting HIV-1. Optionally, the compound can be a nonnucleoside reverse transcriptase inhibitor.

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The compositions of the invention can be administered *in vivo* in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable. Thus, the material may be administered to a subject, without causing undesirable biological effects or interacting
5 in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art.

The compositions identified by the methods disclosed herein can be
10 administered orally, parenterally (e.g., intravenously), by intramuscular injection, intravenously, subcutaneously, intramuscularly, by intraperitoneal injection, transdermally, extracorporeally, topically or the like, including topical intranasal administration or administration by inhalant. topically or by liposome-mediated delivery. As used herein, "topical intranasal administration" means delivery of the
15 compositions into the nose and nasal passages through one or both of the nares and can comprise delivery by a spraying mechanism or droplet mechanism, or through aerosolization of the small molecule or ligand. Administration of the compositions by inhalant can be through the nose or mouth via delivery by a spraying or droplet mechanism. Delivery can also be directly to any area of the respiratory system (e.g.,
20 lungs) via intubation.

The dosage of the compositions required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the infection being treated, the particular active agent used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for
25 every composition. However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein.

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The materials may be in solution, suspension (for example, incorporated into microparticles, liposomes, or cells). These may be targeted to a particular cell type via antibodies, receptors, or receptor ligands.

Suitable carriers and their formulations are described in *Remington: The Science and Practice of Pharmacy* (19th ed.) ed. A.R. Gennaro, Mack Publishing Company, Easton, PA 1995. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically-acceptable carrier include, but are not limited to, saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8.5, and more preferably from about 7.8 to about 8.2. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of composition being administered.

Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration of drugs to humans, including solutions such as sterile water, saline, and buffered solutions at physiological pH. Other compounds will be administered according to standard procedures used by those skilled in the art.

Pharmaceutical compositions may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the molecule of choice. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, anti-inflammatory agents, anesthetics, and the like.

The terms "effective amount" and "effective dosage" are used interchangeably. The term "effective amount" is defined as any amount necessary to produce a desired

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physiologic response. Effective amounts and schedules for administering the compositions may be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for the administration of the compositions are those large enough to produce the desired effect in which the symptoms or disorder are affected. The dosage should not be so large as to cause substantial adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex, type of disease and extent of the disease in the patient, route of administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any contraindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products.

Parenteral administration of a nucleic acid or vector to a subject is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein. For additional discussion of suitable formulations and various routes of administration of therapeutic compounds, see, e.g., *Remington: The Science and Practice of Pharmacy* (19th ed.) ed. A.R. Gennaro, Mack Publishing Company, Easton, PA 1995.

Also, provided are kits for screening for compounds comprising a plasmid which expresses a fusion protein comprising a p66 subunit, a p51 subunit, and Vpr, and a reverse transcriptase deficient proviral DNA. Also provided are kits comprising a cell comprising the plasmid. Also provided are kits for treating viral infections

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comprising a composition identified by the methods disclosed herein.

The present invention is more particularly described in the following examples, which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

5 Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

10 Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

15 The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless
20 indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

EXAMPLES

Example 1: Subunit-Specific Analysis of the Human Immunodeficiency Virus Type-1 Reverse Transcriptase *In Vivo*

25 *Expression and virion incorporation of heterodimeric RT in trans*

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For independent expression of the p51 and p66 subunits, the bicistronic pLR2P-vpr-p51-IRES-p66 (*vpr-p51/p66*) expression plasmid (abbreviations can be found in Table III) was constructed (Figure 1A). The p51-containing DNA fragment was fused in frame with that of *vpr*. The Vpr-p51 fusion included the natural PR-RT cleavage site (PC), allowing processing by the viral protease and liberation of Vpr (Wu et al., 1997). The encephalomyocarditis virus internal ribosome entry site (IRES) was placed downstream of *vpr-p51*, followed by the p66 coding sequence. Transcription of *vpr-p51/p66* was under control of the HIV-2 LTR (Wu et al., 1995).

TABLE III. Abbreviations for plasmids used in study

Plasmid	Abbreviation
pSG3 ^{WT}	SG3
pSG3 ^{S-RT}	S-RT
pSG3 ^{FN}	FN
pSG3 ^{M7}	M7
pLR2P-vpr-p66	<i>vpr-p66</i>
pLR2P-vpr-Δp51-IRES-p66	<i>vpr-Δp51/p66</i>
pLR2P-vpr-p51-IRES-p66	<i>vpr-p51/p66</i>
pLR2P-vpr-p51-IRES-	<i>vpr-p51/p66^{NN}</i>
p66 ^{YMNN}	
pLR2P-vpr-p51 ^{YMNN} -IRES-	<i>vpr-p51^{NN}/p66</i>
p66	
pLR2P-vpr-p51 ^{YMAA} -IRES-	<i>vpr-p51^{AA}/p66</i>
p66	
pLR2P-vpr-p51 ^{YMEE} -IRES-	<i>vpr-p51^{EE}/p66</i>
p66	

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pLR2P-vpr-p51 ^{YMKK} -IRES-	<i>vpr-p51^{KK}/p66</i>
p66	
pLR2P-vpr-IN	<i>vpr-IN</i>

The proviral clone pSG3^{FN} (FN) (Figure 1B) was used to study incorporation of the heterodimeric *trans*-RT into virions when coexpressed with the *vpr-p51/p66* expression plasmid. The FN clone was selected for this purpose since it contains a deletion in RT that includes most of the RNase H region and extends 13 amino acids into the carboxyl-terminus of the p51 domain. This created a defective RT, while the *pol* reading frame, including IN, remained open. This overall strategy for studying subunit-specific RT function in the context of infectious virus is illustrated in Figure 2. Effective *trans*-complementation would require expression of the two subunits (Vpr-p51 and p66), dimerization and stable association of the p51 (Vpr-p51) and p66 subunits within the cytosol of the cell, specific interaction of Vpr with Pr55^{Gag}, incorporation of the Vpr-p51/p66 heterodimeric complex into virions, proteolytic cleavage to liberate Vpr from p51/p66, and proper interaction of RT with the template-primer.

It was first determined whether the Vpr-p51 fusion protein could selectively incorporate p66 into virions. Virions derived by cotransfecting 293T cells with *vpr-p51/p66* and FN were analyzed by immunoblot analysis. Using polyclonal anti-RT antiserum, two predominant proteins detected were consistent with the molecular masses of p51 and p66 (Figure 3A, lane 6), and comigrated with those detected using SG3 virions (lane 1). Neither protein was detected using the RT-minus pSG3^{S-RT} (S-RT) virus (lane 2). Detection of the 51 kDa polypeptide with polyclonal anti-RT antibody showed that Vpr-p51 was packaged and processing by the viral protease liberated p51. The detection of the 66 kDa polypeptide showed incorporation of p66, however, the molecular mass of the unprocessed Vpr-p51 fusion protein is similar to

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that of p66. Therefore, a monoclonal antibody specific to the RNase H domain of p66 was used as a probe and confirmed incorporation of the *trans*-p66 subunit into virions (Figure 3B, lane 6). As controls, virions produced by transfecting 293T cells with FN alone and FN in combination with the pLR2P-vprRT (*vpr-p66*) expression plasmid were analyzed. A protein comigrating with p51 that likely represents the truncated RT protein product (p51Δ13) was detected in virions generated by FN (lane 3). When the *vpr-p66* expression plasmid was cotransfected with FN, p66, p51 and unprocessed Vpr-p66 were detected in virions (lane 4). To determine whether the incorporation of p66 was mediated selectively by the Vpr-p51 fusion protein, virus derived by cotransfecting 293T cells with FN and pLR2P-vpr-Δp51-IRES-p66 (*vpr-Δp51/p66*) was analyzed (lane 5). The *vpr-Δp51/p66* expression plasmid abrogates expression of the p51-coding region without affecting p66 expression. Using both polyclonal and monoclonal antibodies, a protein with a molecular mass equal to that of p66 was detected, showing that p66 incorporation, at least in part, was not selectively mediated by Vpr-p51 (lane 5). Immunoblot analysis using a monoclonal antibody against CA confirmed that approximately the same amount of each virus was analyzed (Figure 3C).

Specific packaging of heterodimeric RT

The strategy used for analyzing RT subunit function necessitates Vpr-p51-mediated selective incorporation of p66. Non-specifically packaged p66 can form p66/p66 homodimers and through proteolytic processing generate p51/p66 RT heterodimers, thus confounding the analysis of subunit-specific mutations. One possible explanation for the non-specific packaging of p66 observed in Figure 3 was translational read-through of the TAA stop codon placed at the 5' end of p51 in the *vpr-Δp51/p66* expression plasmid. A second possibility was that the *trans*-p66 protein may associate intracellularly with the Gag-Pol polyprotein encoded by FN. Therefore, the pSG3^{M7} (M7) proviral clone was constructed. M7 has multiple mutations in the

RT and IN coding regions (Figure 4A) and was constructed to minimize the chance of encoding functional RT and IN, including that which conceivably could be generated via intermolecular genetic recombination with the *vpr-p51/p66* plasmid. Virus generated by cotransfection of M7 with *vpr-p51/p66* contained the p51 and p66 proteins, detectable with monoclonal anti-RT antibody (Figure 4B, lane 5). In contrast to virus generated by FN (Figure 3), virus generated by cotransfecting 293T cells with M7 and *vpr-Δp51/p66* did not contain detectable p66 (lane 4). Probing blots with p66 monoclonal antibody confirmed selective, Vpr-p51-mediated packaging of p66 (Figure 4C, lane 5). By probing a replica blot with monoclonal antibody against CA, it was confirmed that approximately the same amount of each virus was analyzed, and that the M7 virus did not have detectable abnormalities in either virion assembly or maturation (Figure 4D). These results demonstrated that the Vpr-p51 fusion protein can selectively incorporate the p66 RT subunit into HIV-1 virions. Moreover, they indicate that the p51/p66 heterodimer is relatively stable, subsequent to virion incorporation. If not, free p66 might be expected to form homodimers that would be processed by viral PR, resulting in excess p51. Figure 4B shows that a similar amount of each subunit was present in the M7 virions.

Heterodimeric trans-RT rescues the infectivity of RT-deficient virus

To determine if the heterodimeric *trans*-RT was functional, the M7 proviral construct was cotransfected into 293T cells with *vpr-p51/p66* and *vpr-IN*. The *vpr-IN* expression plasmid was included since the M7 clone does not express the IN protein and integration of the nascent viral cDNA is required to detect infection using the TZM-bl reporter cell line. Moreover, IN is also required for efficient initiation of reverse transcription (Wu et al., 1999). In three independent experiments, virus infectivity was rescued to about 15% of that of wild-type virus (Figure 5, lane 5). Virus derived by cotransfecting 293T cells with M7, *vpr-p66* and *vpr-IN* exhibited a similar level of infectivity (lane 3), consistent with earlier reports (Wu et al., 1997).

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The infectivity of M7 virus derived by cotransfection with *vpr-Δp51/p66* and *vpr-IN* was less than 0.05% of wild-type virus (lane 4), or 0.2% compared with virus complemented with *vpr-p51/p66*. These results demonstrated that the heterodimeric *trans*-RT is functional, and with the M7 proviral clone, minimal complementation of virus infectivity was due to non-Vpr-p51 mediated packaging of p66. It is also interesting to note that virus infectivity was not efficiently complemented without the IN protein (lane 6).

Subunit-specific analysis of the YMDD motif

There exists a preponderance of evidence from biochemical and structural studies that suggests HIV-1 reverse transcription is catalyzed by the p66 subunit of RT. However, the function of D185 and D186 in the p51 and p66 subunits, respectively, has not been directly tested in the context of an infectious virus. To study the function of these aspartate residues in one subunit independently of the other, either the p66 or the p51 coding region of the *vpr-p51/p66* plasmid was mutated in both aspartates of the YMDD motif. Virus was analyzed for infectivity using the TZM-bl reporter cell line and for DNA synthesis following acute infection of JC53 cells. Virus containing the p51/p66^{YMNN} mutant RT with Asp185Asn and Asp186Asn mutations in p66 was severely defective in infectivity (Figure 6A, lane 3). Analysis of infected cells for viral DNA revealed a severe defect in reverse transcription (Figure 6B and C, lanes 5). The severity of this defect, suggested that the p51 subunit of the heterodimer does not catalyze viral DNA synthesis in vivo. Moreover, when the equivalent catalytic site mutation was analyzed in p51 (p51^{YMNN}/p66), virus infectivity was reduced to approximately 70% of that of p51/p66 (wild-type) complemented virus (Fig 6A, lane 4). Similarly, viral DNA synthesis of virus containing the p51^{YMNN}/p66 RT was also modestly reduced compared to that of wild-type (Figure 6B and C, lanes 6). This suggested that the YMDD aspartates of p51 affect viral DNA synthesis.

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To further analyze the role of these p51 aspartates, they were mutated to alanines, glutamates, or lysines. Virus stocks containing each mutant RT were prepared by cotransfection and analyzed for infectivity and DNA synthesis. Similar to the asparagine mutations, the glutamic acid mutations (p51^{YMEE}/p66) decreased virus infectivity and DNA synthesis only slightly (Figure 6). More dramatic decreases in both DNA synthesis and virus infectivity were observed for viruses containing either the alanine (p51^{YMAA}/p66) or the lysine (p51^{YMKK}/p66) p51 mutations. The effect of each of the p51 YMDD mutants on viral DNA synthesis was examined using primer pairs that detect either early (R-U5) or late (R-gag) products of reverse transcription. The magnitude of the defect was similar with both primer pairs, showing that the defect was at or prior to initiation. The cellular expression of Vpr-p51 and p66 by these *trans* Vpr-RT constructs was equivalent, ruling out expression defects as the cause for differences in viral infectivity and DNA synthesis. The effect of each of these mutations on DNA synthesis and infectivity correlate with the disruptiveness of the mutation introduced. This shows that the YMDD motif of p51, specifically its aspartate residues, is important to maintain the structure of the RT heterodimer and its enzymatic function *in vivo*.

Discussion

Vpr-p51 and p66 form an intracellular dimer (Vpr-p51/p66) that is specifically incorporated into virions, processed by the viral PR to liberate p51/p66, and rescues the infectivity of RT-deficient HIV-1. By analyzing mutations in the YMDD aspartates of either p51 or p66 the function of these residues in the context of an infectious virus was delineated. The absence of minus-strand strong-stop DNA synthesis in cells infected with virus, in which the YMDD aspartates of p66 were mutated, corroborates findings from previous *in vitro* studies, and demonstrates that in a heterodimer, p66 is solely responsible for the catalytic/polymerase function of RT *in vivo*. The analysis of the p51 subunit indicates that mutations in the YMDD aspartates

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impair virus infection and DNA synthesis due to an effect on RT structure rather than catalytic function.

The YXDD motif (SEQ ID NO: 9) of retroviral RTs is highly conserved and it has been described in the active site of many viral and cellular polymerases (Kamer and Argos, 1984; Toh et al., 1983). The HIV-1 YMDD motif is situated in the palm domain (Ding et al., 1998; Kohlstaedt et al., 1992; Sarafianos et al., 2001). The Y183 and M184 amino acid residues contribute to the dNTP binding pocket of p66 (Huang et al., 1998). While some substitutions of these residues are tolerated, most mutations at these sites reduce polymerase function (Lowe et al., 1991; Wakefield et al., 1992).

10 The most conserved feature of the YMDD motif is the aspartates (D185 and D186), which together with a third aspartate (D110) form the polymerase catalytic triad. Mutation of the aspartates abolishes RT catalytic function and virus infectivity (Boyer et al., 1992; Larder et al., 1987b; Lowe et al., 1991). The role of the catalytic aspartates in each RT subunit has been studied by expressing p51 and p66 separately

15 in *Escherichia coli* (Hostomsky et al., 1992; Le Grice et al., 1991). Recombinant heterodimers containing polymerase active site mutations exclusive to p51 retain almost wild-type levels of polymerase activity, whereas heterodimers containing the same mutation(s) in p66 appear to be defective in polymerase activity. In the RT heterodimer, the polymerase domain of p51 assumes a closed conformation

20 (Kohlstaedt et al., 1992), and therefore p51 does not appear to play a catalytic role in reverse transcription *in vivo*.

The role of the p51 YMDD aspartates in reverse transcription was investigated by analyzing the effects that different mutations had on reverse transcription and virus infectivity. The p51 YMDD aspartates were substituted with both conservative and

25 non-conservative amino acid residues. The YMNN mutant is relatively conservative, since asparagine is almost isosteric to, but less charged than, Asp. In the YMEE mutant the length of the side chain is increased by one methylene group without

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changing the negative charge. Similar to aspartate, asparagine and glutamine are capable of participating in hydrogen bond interactions through their side chains. These two p51 mutants caused a slight reduction of infectivity and DNA synthesis. Substitution of the p51 YMDD aspartates with either alanines (YMAA) or lysines
5 (YMKK) drastically reduced infectivity and DNA synthesis. The alanines have a short hydrophobic side chain that cannot make hydrogen bonds with neighboring polar residues. The lysines present an opposite polarity through a lengthened side chain. Small changes in charge and/or length of the side-chain can be tolerated (i.e. YMNN and YMEE), however, a charge shift and/or substantial changes in side chain length
10 are not (i.e. YMKK and YMAA). These findings suggest that side chain interactions of p51 YMDD aspartates are important for RT function.

The D185 and D186 residues of p51 YMDD are within interacting distance (approximately 3 Å) of residues T409 and W410 of the p51 connection subdomain. The T409 and W410 residues lie in the loop between alpha helix L (α L) and beta
15 sheet 20 (β 20). This loop is a part of the putative "tryptophan motif" (Trp-motif) of the p51 connection subdomain, which is believed to be critical for p51-p66 dimerization (Baillon et al., 1991; Divita et al., 1994; Tachedjian et al., 2003). It is plausible that mutation at the p51 YMDD aspartates cause repositioning of the α L- β 20 loop, which in turn could affect multiple interactions involving the Trp-motif and
20 the heterodimer interface. The orientation of the α L- β 20 loop could also influence template binding as these residues are in the proximity of the floor of the DNA binding cleft/RNase H primer grip, which includes K390, K395 and E396 of p51 that interact directly with the template-primer (Huang et al., 1998; Sarafianos et al., 2001). Mutation of the p51 YMDD aspartates may also affect intermolecular interactions that
25 maintain its structure in the RT heterodimer. In the p51 subunit, the connection subdomain folds into the expanded cleft between its fingers and thumb subdomains, which gives it a "closed" conformation. Since the connection subdomain of p51 makes multiple contacts with the three other subdomains of p51 (fingers, palm and thumb),

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destabilization of the interaction between the α L- β 20 loop and YMDD can have global effects on RT folding. In addition to the interactions with T409 and W410, the YMDD motif is buried within the core of p51, and thus, the aspartates could interact with other neighboring residues. These include interdomain interactions between
5 D185/186 and R72 of the p51 fingers subdomain; D185 and Q151-G152 at the tip of α helix E in the palm subdomain and D185/D186 with the tryptophan-rich region of p51 (Figure 7).

The finding that subunit-specific analysis of RT function can be studied using infectious virus has broad implications. While the p51 subunit was believed to
10 function primarily as a scaffold to maintain the active structure of p66 (Hughes, 2001; Telesnitsky and Goff, 1997), other functions have been suggested, including involvement in tRNA primer-binding (Arts et al., 1994; Jacques et al., 1994), loading of p66 onto the template-primer (Harris et al., 1998) and enhancement of strand displacement (Amacker et al., 1995; Hottiger et al., 1994). The dimer interface
15 between p51 and p66 is critical for reverse transcription and it has been proposed as an ideal target for therapeutic intervention (Divita et al., 1994; Morris et al., 1999; Restle et al., 1990). This notion was supported by several studies demonstrating that mutation of amino acid residues involved in subunit interactions alter the arrangement of the RT subdomains and disrupt RT function (Ghosh et al., 1996; Menendez-Arias
20 et al., 2001; Tachedjian et al., 2003). Mutations in p51 have been also implicated in resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs) and inhibitors of RNase H activity. The E138K mutation, which confers resistance to TSAO {2',5'-Bis-O-(tert-butyldimethylsilyl)-3'spiro-5''-(4''-amino-1'',2''-oxathiole-2'',2''-dioxide)}

has been mapped to the p51 subunit (Jonckheere et al., 1994; Sluis-Cremer et al.,
25 2000). The C280S mutation in RT causes resistance to the RNase H inhibitor N-ethylmaleimide (NEM) (Loya et al., 1997). Interestingly, both the p51 and p66 subunits were found to contribute to the resistance of the enzyme to NEM *in vitro*.

Materials and Methods

Cells and Antibodies

The 293T, JC53 (Platt et al., 1998), and TZM-bl cell lines (Wei et al., 2002) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplement with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). The anti-RT antiserum (R1465) was generated against HIV-1 RT expressed in *E. coli*. Briefly, the entire RT coding region of HIV-1/pSG3 was ligated into the prokaryotic pGEX expression vector (pGEX-RT). *E. coli* (DH5 α) were transformed with pGEX-RT and protein expression was induced with isopropyl β -D-thiogalactopyranoside (IPTG). Expression of the glutathione S-transferase- (gst) RT fusion protein was confirmed by SDS-PAGE. Soluble gst-RT protein was purified and RT was released by thrombin cleavage using previously described procedures (Smith and Johnson, 1988). New Zealand white female rabbits were immunized subcutaneously with 200 μ g of purified RT protein emulsified in an equal volume of Freund's complete adjuvant. Rabbits were boosted at two week intervals with 200 μ g of RT mixed with an equal volume of Freund's incomplete adjuvant. Sera were tittered and analyzed for specificity by immunoblotting against purified preparations of both the immunizing protein and concentrated HIV-1 virions. Additional antibodies used in these studies included monoclonal antibodies to HIV-1 capsid (183-H12-5C, contributed by Dr. Bruce Chesebro and Dr. Hardy Chen) and HIV-1 RT (8C4 and 7E5, contributed by Dr. Dag E. Helland), obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

HIV-1 proviral clones

The HIV-1 pSG3 proviral clone (SG3) (Ghosh et al., 1993) (Genbank Accession # L02317) was used to produce wild-type virus, and to construct RT deficient proviral clones and all recombinant RT and IN expression plasmids. The

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pSG3^{FN} (FN) clone was constructed using the strategy described by Dubay *et. al.* (Dubay *et al.*, 1992) for the HXB2 pFN clone (Figure 1B). Briefly, the FN clone contains an in-frame 110 amino acids deletion and was created by *Acc65I* digestion to remove a 330-nucleotide fragment of the *pol* gene. The 5' overhang was filled using
5 dGTP and the Klenow fragment of DNA polymerase. The remaining single-stranded regions were removed with S1 nuclease and the plasmid was religated. The deleted DNA segment encoded a large part of RNase H and 13 amino acids of the carboxyl-end of the polymerase domain of RT. This clone encodes a truncated form of RT while maintaining the IN coding region in-frame.

10 The pSG3^{M7} (M7) proviral construct was created from pSG3^{S-RT} (S-RT) (Wu *et al.*, 1997). In addition to stop codons in the RT and IN coding regions of pSG3^{S-RT}, M7 contains three additional stop codons at amino acid positions 441 (TAA), 444 (TGA) and 447 (TAG) and a D443N RNase H catalytic mutation in the RNase H reading frame. The primers (sense [5'-AAGCCCGGGATGGATGGCCCAAAGT-
15 3'], SEQ ID NO: 10 and antisense [5'-TCCTAAACGCGTCTCCCTCTAAGCTGCTCAATTTACTTAGAAAGT-3'], SEQ ID NO: 11) containing *XmaI* and *MluI* sites, respectively, and the primers (sense [5'-ACTTTCTAAGTAAATTGAGCAGCTTAGAGGGAGACGCGTTTAGGA-3'] (SEQ ID NO: 12) and antisense [5'-TATGTCGACACCCAATTATGAAAAG-3']
20 (SEQ ID NO: 13)) containing *MluI* and *SaII* sites, respectively, were used to amplify two DNA fragments from the S-RT constructs (nucleotides 2132-3455 and 3410-5340). The *XmaI-MluI* and *MluI-SaII* PCR products were digested with corresponding restriction endonucleases, purified and ligated together into an *XmaI-SaII* cut pSG3^{S-RT} plasmid.

25 *Construction of heterodimeric RT expression plasmid*

To express the RT subunits in trans with RT-minus proviral DNA, the pLR2P-vpr-p51-IRES-p66 (*vpr-p51/p66*) expression plasmid was constructed. Briefly, the

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- sense [5'-TAGATCAGATCTGTTGACTCAGATTGGTTGCA-3'] (SEQ ID NO: 14) and antisense [5'-ATCTACACGCGTTTAGAAGGTTTCTGCGCCTT-3'] (SEQ ID NO: 15) primers containing the *Bgl*III and *Mlu*I restriction sites (underlined), respectively, were used to PCR amplify a p51-containing DNA fragment from pSG3.
- 5 The internal ribosome entry site (IRES) was PCR amplified from the encephalomyocarditis virus (EMCV) (Duke et al., 1992) (Genbank Accession # NC_001479) using the sense ([5'-
- TTATTAACGCGTCCGCCCCTCTCCCTCCCCC-3'] (SEQ ID NO: 16) and antisense [5'-
- 10 CCATCCCGGGCTTTAATTTTACTGGTACAGTTTCAATAGGACTAATGGGTC CCATGGTATTATCGTCTT-3'] (SEQ ID NO: 17) primers containing *Mlu*I and *Xma*I sites (underlined), respectively. The PCR-derived p51 fragment was digested with *Bgl*III and *Mlu*I, while the IRES fragment was digested with *Mlu*I and *Xma*I. These two fragments were ligated simultaneously into the *Bgl*III-*Xma*I-cut pLR2P-
- 15 vprRT (Wu et al., 1997), generating pLR2P-vpr-p51-IRES-p66. This construction strategy (Figure 1A) placed *vpr* and *RT* in-frame, while preserving the N-terminal protease cleavage (PC) site of RT by including 33 bps of PR sequence 5' of RT. The antisense primer introduced a translational stop codon (TAA) to terminate RT expression at amino acids 440, which is the full-length p51 subunit. The vpr-p51
- 20 reading frame was followed by the IRES and then p66. To enable efficient expression of p66, an artificial Kozak sequence was included at the 5' of the p66 coding sequence (Kozak, 1987). This modification added a Met-Gly onto the p66 N-terminus. The pLR2P-vpr-Δp51-IRES-p66 (*vpr-Δp51/p66*) control plasmid was constructed to contain a translational stop codon at the first amino acid position of p51 by
- 25 amplification of a *Bgl*III-*Mlu*I DNA fragment from the S-RT clone. Other derivatives of *vpr-p51/p66* were constructed using PCR based site-directed mutagenesis, restriction digestion with the appropriate enzyme, and cloning into the *Bgl*III-*Mlu*I or *Xma*I-*Xho*I sites for p51 or p66 mutant clones, respectively. All clones were

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confirmed by sequencing. The pLR2P-vprIN (*vpr-IN*) expression vector has been described previously (Wu et al., 1997).

Transfections and analysis of virus infectivity

DNA transfections were performed on monolayer cultures of 293T cells grown
5 in 6-well plates using the calcium phosphate DNA precipitation method. Unless
otherwise noted, each cell monolayer (well) was transfected with 6 µg of proviral
DNA, 3 µg of the *vpr-p51/p66* constructs and 1 µg of the *vpr-IN* constructs. Culture
supernatants from the 293T cells were collected 60 h post-transfection, clarified by
low-speed centrifugation (1000 x g, 10 min), and filtered through 0.45 µm pore-size
10 sterile filters. The clarified supernatants were analyzed for HIV-1 p24 antigen
concentration by ELISA (Beckman-Coulter Inc.).

Virus infectivity was assessed using the TZM-bl reporter cell line as described
earlier (Wei et al., 2002). Briefly, virus containing supernatants were normalized for
p24 antigen concentration, serially diluted (five-fold dilutions) and used to infect
15 monolayer cultures of TZM-bl cells. At 48 hrs post-infection, the cells were fixed and
stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) reagent as
described earlier (Kimpton and Emerman, 1992). The blue-stained cells were counted
using a light microscope. Wells containing between 30 and 300 blue cells were used
to calculate the infectious units of virus per ng of p24 antigen (IU/p24-ng).

20 *Semiquantitative detection of viral DNA*

The PCR method used to analyze the synthesis of nascent viral DNA in
infected cells was similar to those described earlier (Fassati and Goff, 2001; Zack et
al., 1990). Briefly, 500-ng equivalents (p24 antigen) of transfection-derived virions
were incubated with DNase I (4 µg/ml; Worthington Inc.) at 37°C for 1 hr to
25 minimize plasmid DNA carryover. The treated virus was then used to infect one
million JC53 cells for 4 hrs in DMEM (1% FBS, 10 µg/ml DEAE-dextran). The cells

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were washed twice with DMEM, and the medium was replaced with complete DMEM (10% FBS). At 18 hrs post-infection, the cells were lysed and total DNA was extracted by organic methods, resuspended in 200 µl of distilled water and treated with the *DpnI* restriction endonuclease to digest bacterially derived plasmid DNA.

- 5 Each PCR subjected 250 pgs of DNA extract to 30 rounds of amplification with primers designed to detect early (R-U5 [sense nucleotides 79-99 AGCTTGCCTTGAGTGCTTCAA (SEQ ID NO: 18) and antisense nucleotides 182-157 CTGCTAGAGATTTTCCACACTGACTA] SEQ ID NO: 19) and late (R-gag [sense nucleotides 43-63 GGCTAGCTAGGGAACCCACTG (SEQ ID NO: 20) and
10 antisense nucleotides 355-334 ATACTGACGCTCTCGCACCCAT] (SEQ ID NO: 21)) viral DNA. The PCR products were separated on a 1.0% agarose gel and visualized by ethidium bromide staining. The relative amount of amplified DNA was determined by comparison to known standards (serial dilutions of pSG3 DNA).

Western Blot (immunoblot) analysis

- 15 Transfection-derived virions were concentrated by ultracentrifugation through 20% sucrose cushion (125,000 x g, 2 hr, 4°C) using a SW41 rotor (Beckman Inc.). Pellets were solubilized in loading buffer (62.5 mM Tris-HCl [pH 6.8], 0.2% SDS, 5% 2-mercaptoethanol, 10% glycerol), boiled, and proteins were separated on 12.0% polyacrylamide gels containing SDS. Following electrophoresis, proteins were
20 transferred to nitrocellulose (0.2-µm pore size) by electroblotting and incubated for 1 hr at room temperature in blocking buffer (5% nonfat dry milk in phosphate-buffered saline [PBS]). The blocked blot was exposed to the appropriate primary antibody for 1 hr in blocking buffer with constant mixing. After extensive washing, bound antibodies were detected by chemiluminescence using horseradish peroxidase-conjugated
25 species-specific secondary antibodies (Southern Biotechnology Associates, Inc.) as described by the manufacturer (Amersham Biosciences).

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Example 2: Exogenous Reverse Transcriptase Assay

Disclosed are biochemical assays for determining reverse transcriptase activity. One example of such an assay is the Chemiluminescent Reverse Transcriptase Assay by Roche (Cat. No. 1 828 657, Instruction Manual Version 3, February 2004, herein incorporated by reference in its entirety for its teaching regarding reverse transcriptase assays). The protocol is a non-radioactive enzyme immunoassay useful for highly sensitive, quantitative determination of reverse transcriptase activity by chemiluminescence detection. This assay is designed for highly-sensitive and quantitative determination of RT activity, e.g. in cell cultures and other life science research applications. The assay has been shown to be useful for the determination of RT activity derived from a variety of retroviruses, including HIV-1, HIV-2, SIV-1 and CAEV. The assay can be used to determine the propagation of retroviruses in retrovirus-infected mammalian cell cultures. The assay can also be used for *in vitro* screening for RT inhibitors.

Example 3: p51-IRES-p66 Rescues the Infectivity of RT-IN-Minus Virus (M7)

Regarding Table 4: the table shows that Vpr-p51-ires-p66 rescues the infectivity of RT-IN-minus virus (M7), and viruses derived from proviral DNA containing mutations in the YMDD motif of RT, including YMAA and YMND. Virus derived from proviral DNA and the control pLR2P-vpr is not infectious. Methods: 293T cells were transfected with the indicated plasmid (either viral DNA or trans-RT DNA) DNAs plus the pLR2P-vor-IN expression plasmid DNA. 48 hrs later the supernatant viruses were collected and analyzed for infectivity using the JC53-BL reporter assay.

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Table 4:

Construct Name		Blue Cells		Infectious virions/ml	p24 ngs/ml	Virions/ng	% of SG3
		A		$A/B \times 1000 = C$	D	C/D	
pLR2P-vpr	--	93		#DIV/0!	714	#DIV/0!	100.00
pLR2P-vpr	--	0		0.00E+00	397	0.00E+00	0.00
pLR2P-vpr	--	0		#DIV/0!	688	#DIV/0!	#DIV/0!
vpr-p51-IRES-p66	--	66		#DIV/0!	563	#DIV/0!	#DIV/0!
pLR2P-vpr	--	0		#DIV/0!	382	#DIV/0!	#DIV/0!
vpr-p51-IRES-p66	--	21		#DIV/0!	1072	#DIV/0!	#DIV/0!

Construct Name		Blue Cells	Dilution	Virions/ml	p24 ngs/ml	Virions/ng	% of SG3
		A	B	$A/B \times 1000 = C$	D	C/D	
pLR2P-vpr	--	225	0.2	1.13E+06	714	1.58E+03	100.00
pLR2P-vpr	--	0	5	0.00E+00	397	0.00E+00	0.00
pLR2P-vpr	--	2	5	4.00E+02	688	5.81E-01	0.04
vpr-p51-IRES-p66	--	843	5	1.69E+05	563	2.99E+02	19.01
pLR2P-vpr	--	0	5	0.00E+00	382	0.00E+00	0.00
vpr-p51-IRES-p66	--	66	5	1.32E+04	1072	1.23E+01	0.78

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SEQUENCES

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40 SEQ ID NO: 9
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SEQ ID NO: 10

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SEQ ID NO: 12

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SEQ ID NO: 13

10 5'-TATGTCGACACCCAATTATGAAAAG-3'

SEQ ID NO: 14

5'-TAGATCAGATCTGTTGACTCAGATTGGTTGCA-3']

15

SEQ ID NO: 15

5'-ATCTACACGCGTTTAGAAGGTTTCTGCGCCTT-3'

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SEQ ID NO: 17

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What is claimed is:

1. A cell comprising: (i) a vector comprising a p66 subunit, a p51 subunit, and Vpr, wherein Vpr and p51 are expressed as a fusion protein; (ii) and a reverse transcriptase deficient proviral DNA.
2. A method of screening for a compound that inhibits viral reverse transcriptase comprising: a) contacting the cell of claim 1 with the compound, and b) comparing the level of viral infectivity in the presence of the compound with the level of viral infectivity in the absence of the compound, wherein a decreased level of infectivity in the presence of the compound indicates that the compound inhibits reverse transcriptase.
3. The method of claim 2, wherein the virus is a lentivirus.
4. The method of claim 3, wherein the virus is HIV-1.
5. The method of claim 2, wherein the p51 and p66 subunits are expressed in trans in the cell.
6. The method of claim 2, wherein the p51 and p66 subunits are expressed on different messenger RNAs.
7. The method of claim 2, wherein the p51 and p66 subunits are expressed on the same messenger RNAs.
8. The method of claim 2, wherein expression of Vpr-p51 incorporates p66 protein into viral particles.
9. The method of claim 1, wherein p51 interacts with p66 protein.
10. The method of claim 1, wherein p51 contains a mutation, insertion, or deletion.
11. The method of claim 1, wherein p66 contains a mutation, insertion, or deletion.
12. The method of claim 2, wherein the plasmid also expresses an internal ribosome entry site (IRES).

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13. A method of screening for a compound that inhibits dimerization of a p66 subunit polypeptide of reverse transcriptase and a p51 subunit polypeptide of reverse transcriptase comprising: a) contacting the cell of claim 1 with the compound, and b) comparing the level of complex formation in the presence of the compound with the level of complex formation in the absence of the compound, a lower level of complex formation indicating that the compound inhibits dimerization of the p66 subunit and a p51 subunit.
14. A method of screening for a compound that enhances dimerization of a p66 subunit polypeptide of reverse transcriptase and a p51 subunit polypeptide of reverse transcriptase comprising: a) contacting the cell of claim 1 with the compound, and b) comparing the level of complex formation in the presence of the compound with the level of complex formation in the absence of the compound, a lower level of complex formation indicating that the compound enhances dimerization of the p66 subunit and a p51 subunit.
15. A method of making a pharmaceutical composition which comprises: a) determining whether a compound inhibits reverse transcriptase by the method of claim 2; and b) admixing the compound with a pharmaceutically acceptable carrier.
16. A method of inhibiting viral reverse transcriptase comprising contacting (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of the compound identified by the method of claim 2, thereby inhibiting viral reverse transcriptase.
17. A method of inhibiting dimerization of a p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase, which comprises contacting either (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of the

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compound identified by the method of claim 13, thereby inhibiting dimerization of the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase.

18. A method of enhancing dimerization of a p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase, which comprises contacting either (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of the compound identified by the method of claim 14, thereby enhancing dimerization of the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase.
19. The method of claim 17, wherein the HIV-1 reverse transcriptase is present in a subject.
20. The method of claim 14, wherein the compound is administered orally, intravenously, subcutaneously, intramuscularly, topically or by liposome-mediated delivery.
21. A compound identified by the method of claim 2.
22. A compound identified by the method of claim 13.
23. A compound identified by the method of claim 14.
24. A composition which comprises the compound of claim 21 and a carrier.
25. The compound of claim 21, wherein the compound is capable of inhibiting HIV-1.
26. The compound of claim 25, wherein the compound is a nonnucleoside reverse transcriptase inhibitor.
27. An expression cassette comprising LTR-vpr-p51-IRES-p66.
28. The expression cassette of claim 27, wherein the nucleic acid comprises SEQ ID NO: 1.

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- 29. The method of claim 2, wherein the HIV or SIV particles are derived by genes expressed in the cell and wherein the genes contain one or more nucleotide mutations.
- 30. A transgenic animal expressing vpr-p51/66.
- 31. A cell line comprising an exogenous nucleic acid, the nucleic acid comprising vpr-p51/66.
- 32. The cell line of claim 31, wherein the cell expresses viral nucleic acids.
- 33. The cell line of claim 31, wherein the cell can be induced to express viral nucleic acids by contacting the cell with a stimulus.

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ABSTRACT

This invention relates to methods and compositions for identifying compounds that inhibit HIV-1 subunit-specific reverse transcriptase.

5

Figure 1A-B

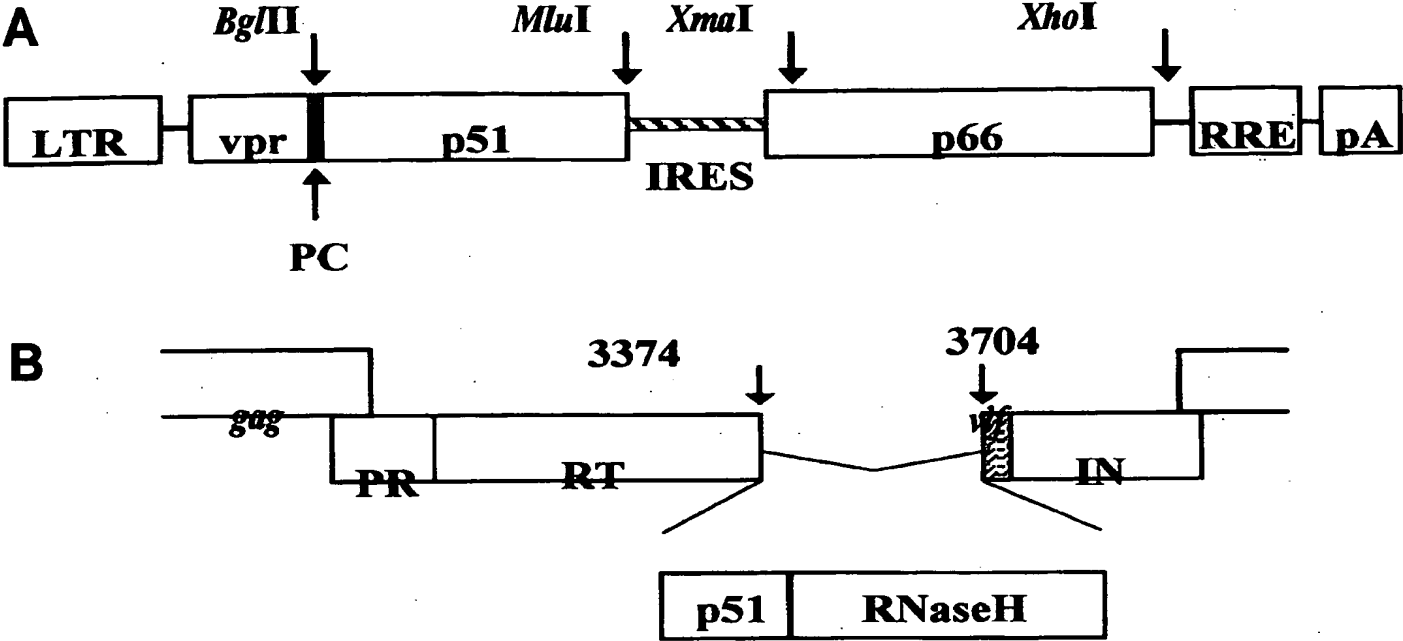


Figure 2

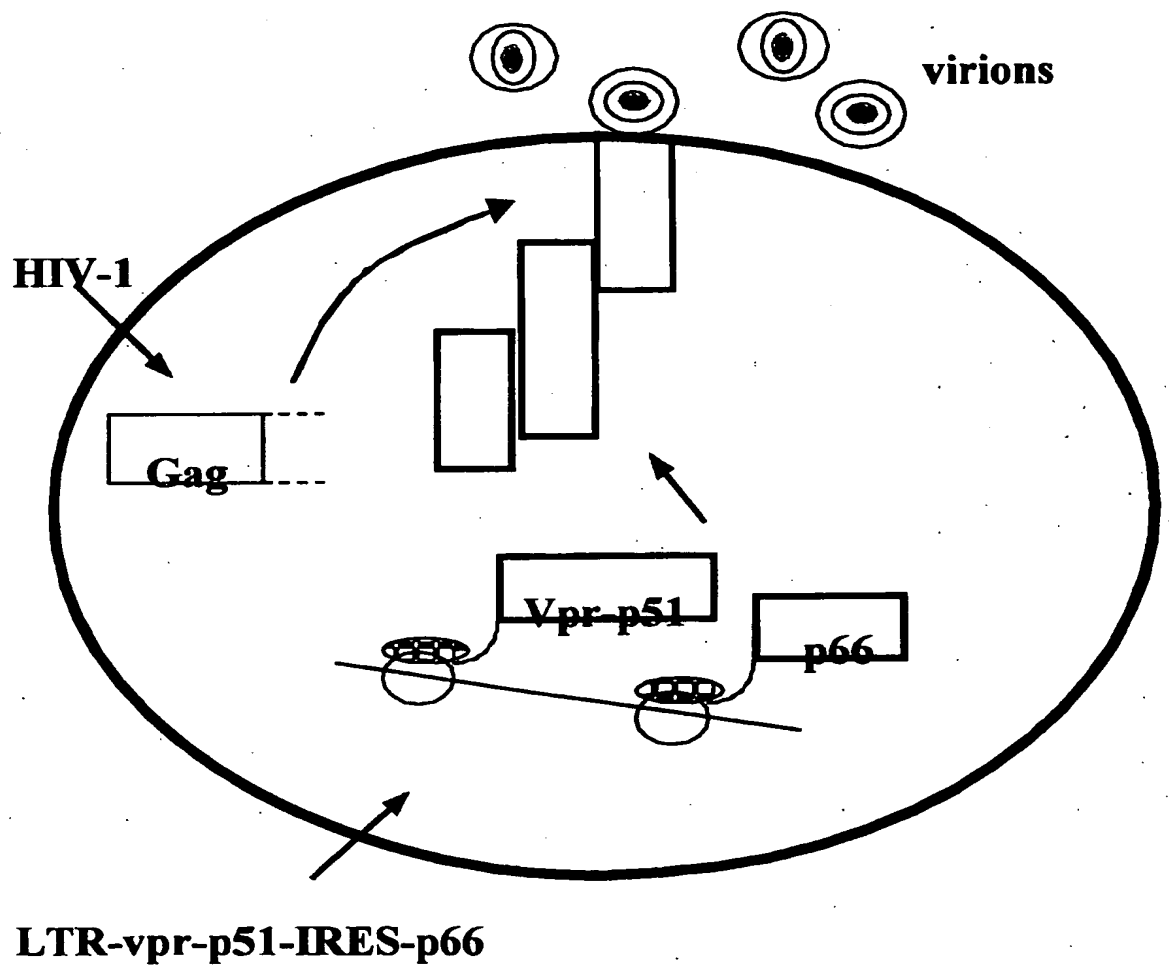


Figure 3

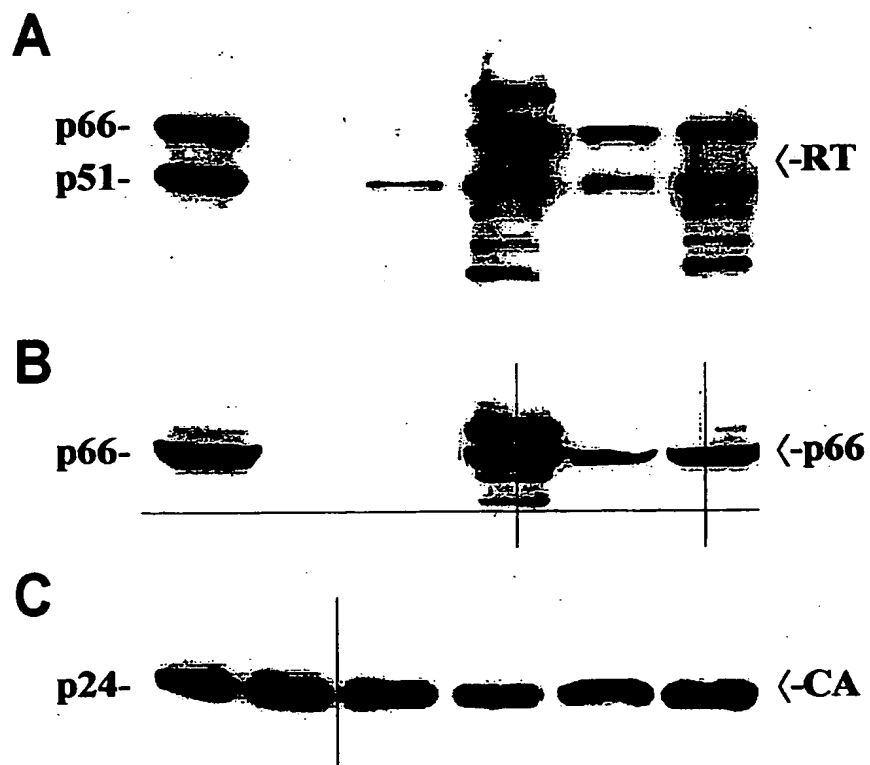


Figure 4

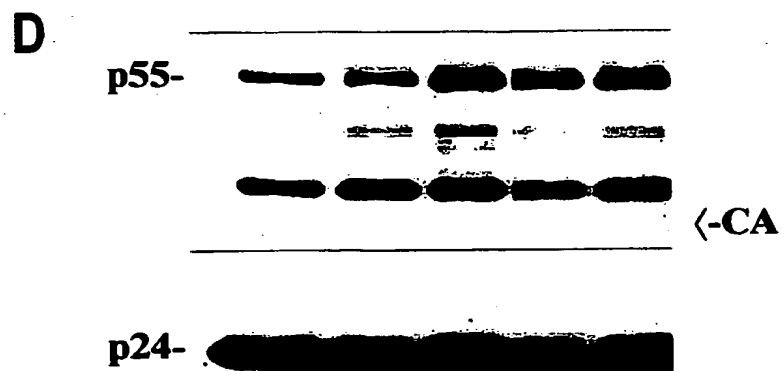
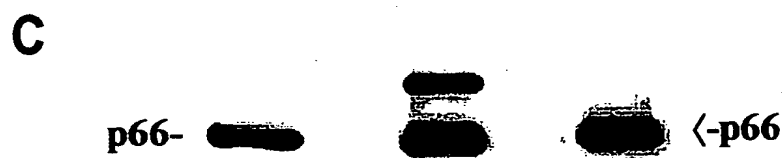
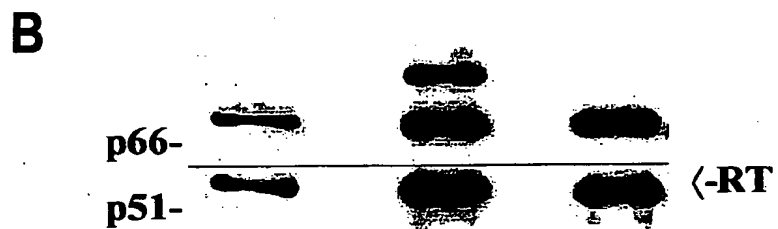
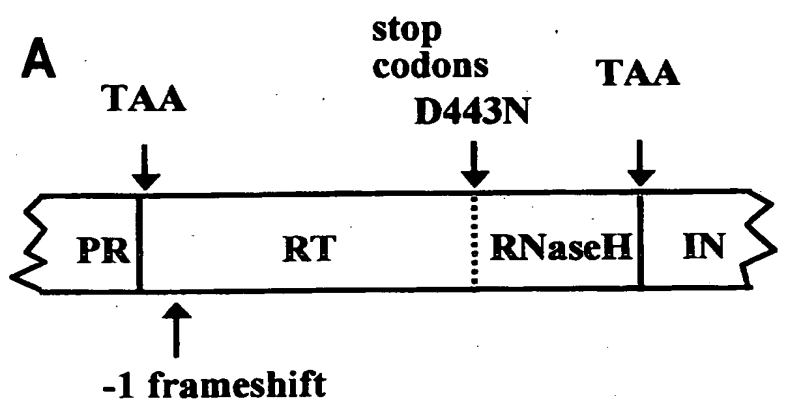
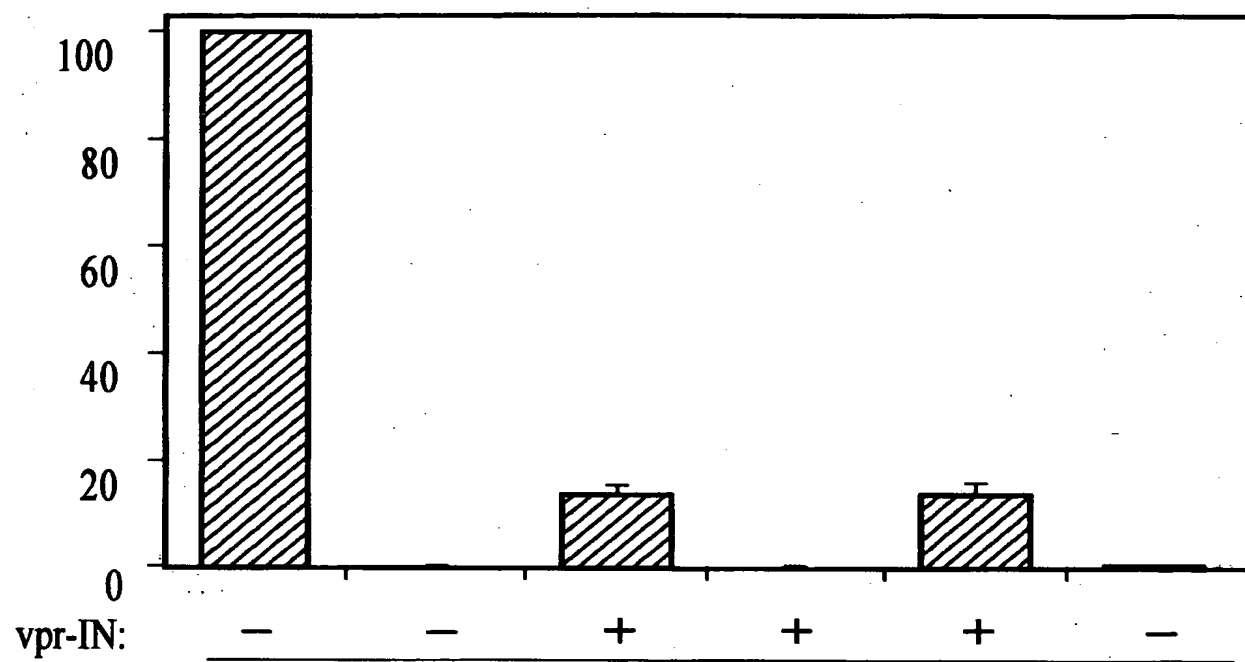
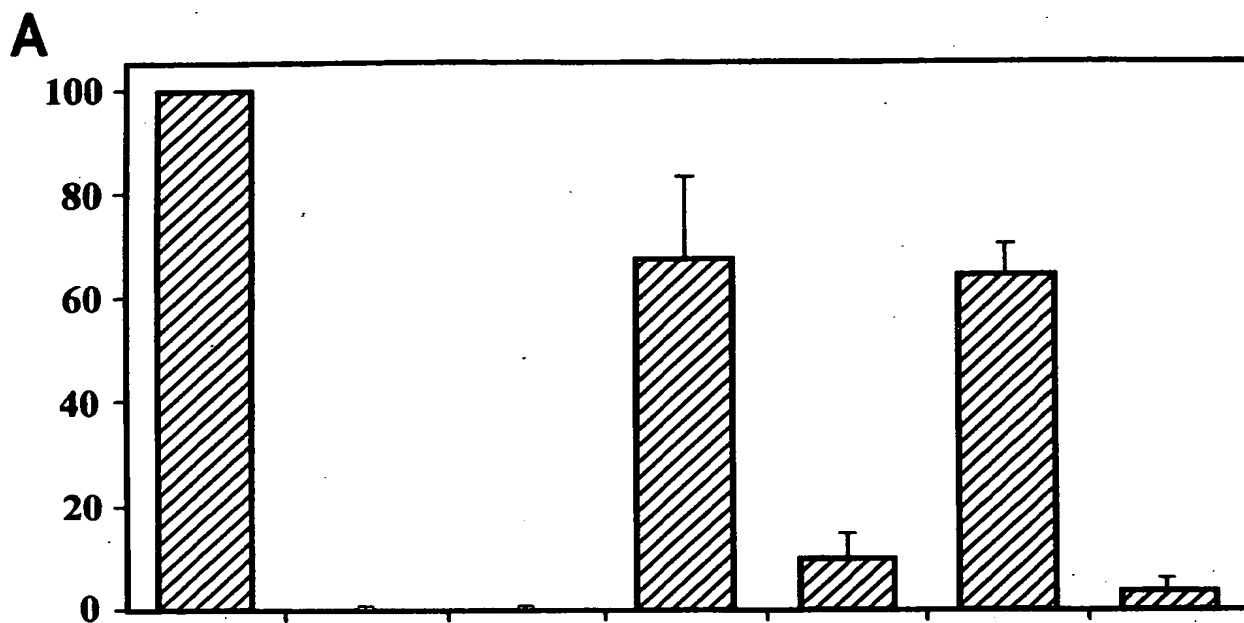


Figure 5





copies of pSG3

Figure 6

Figure 7

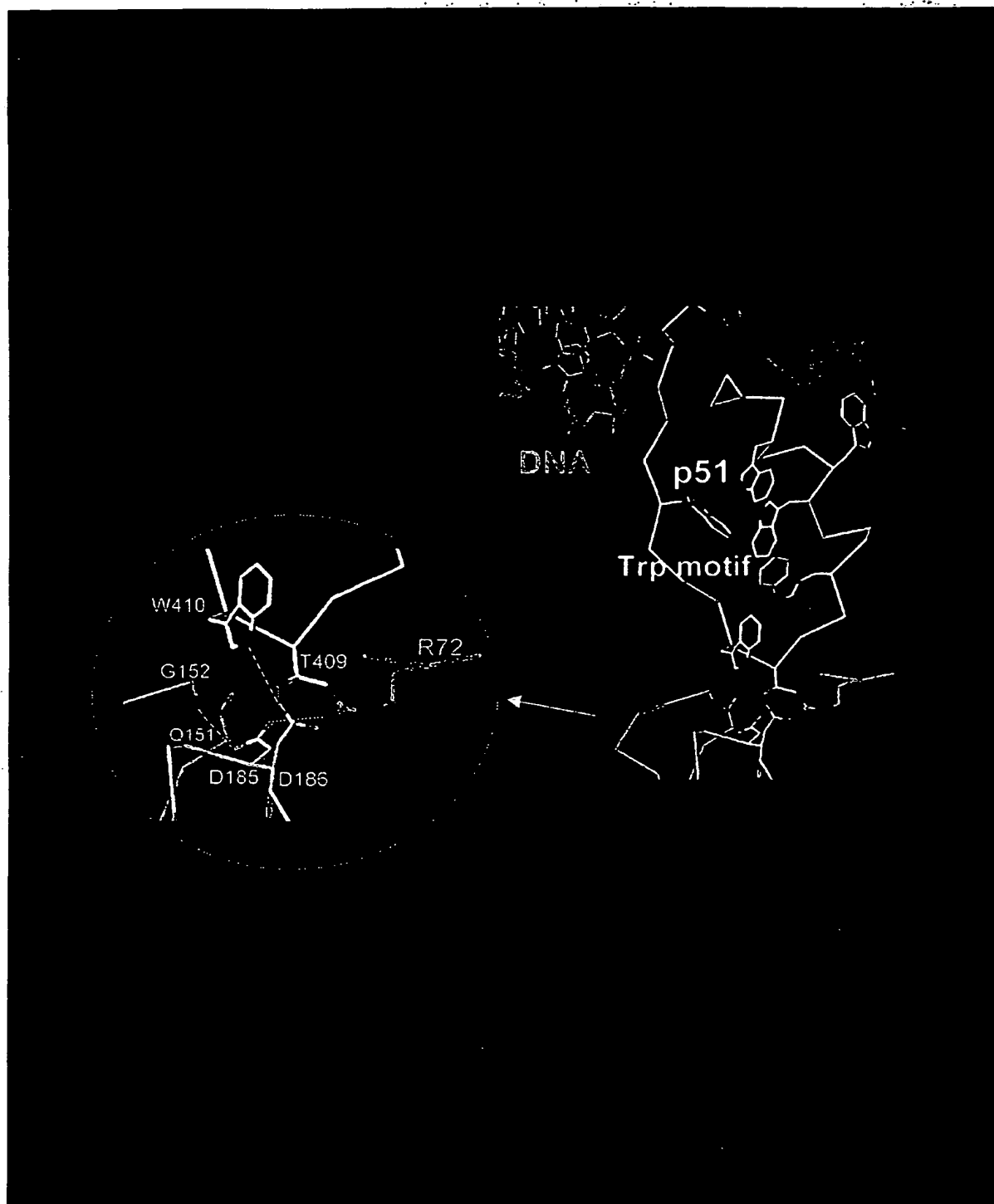


Fig 8A. Infectivity for Trp motif mutants

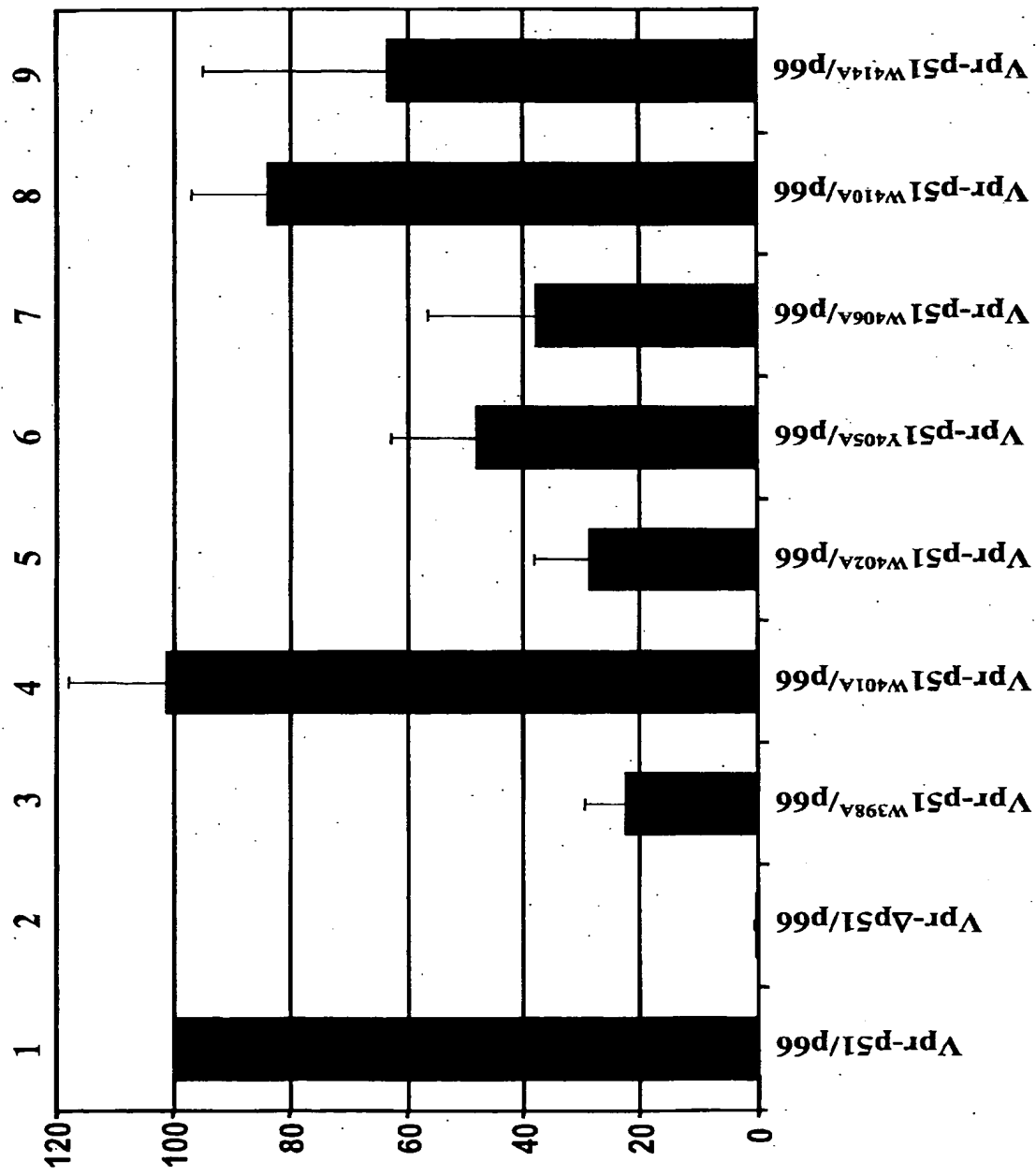


Fig 8B. RT assay for Trp motif mutants

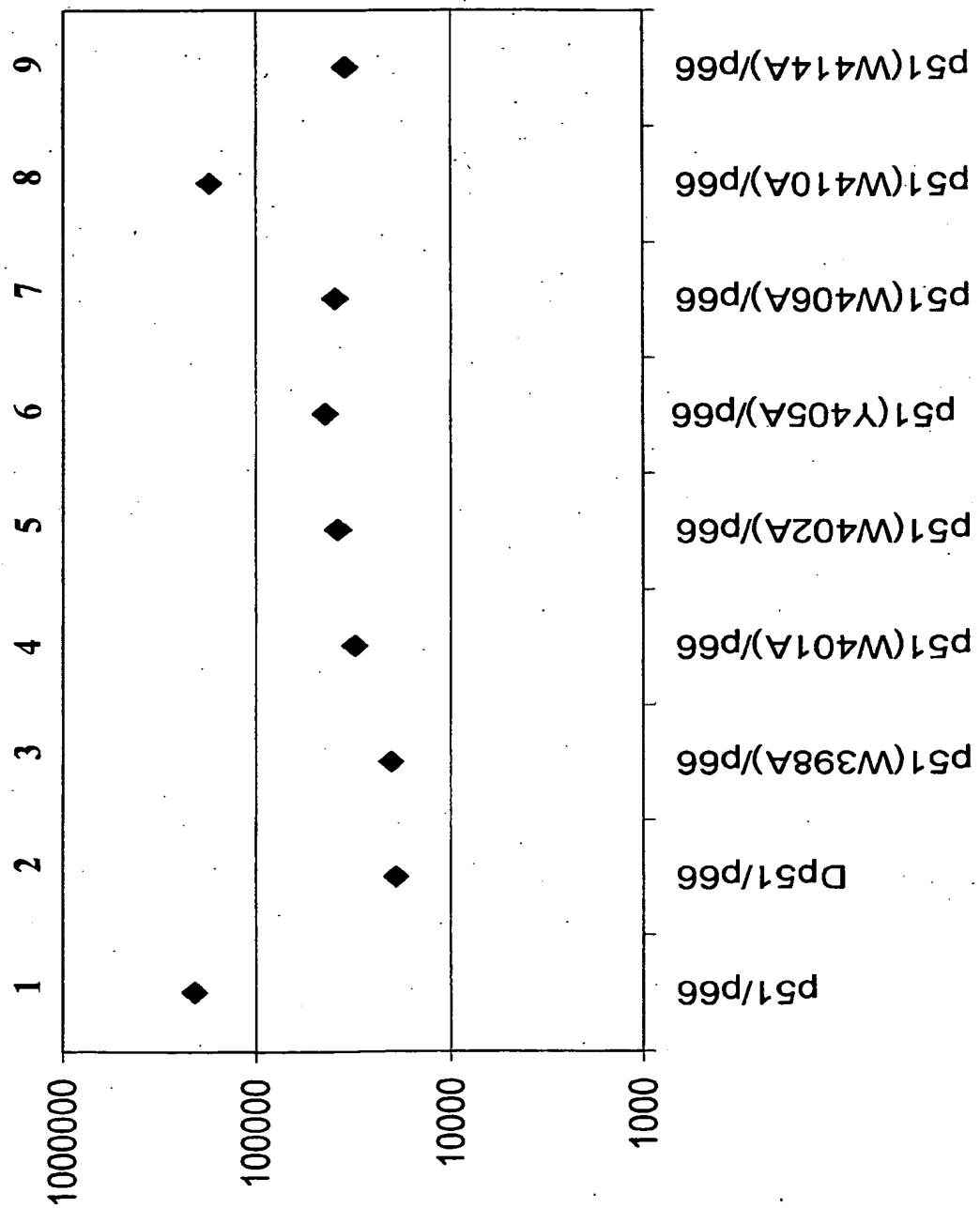


Fig 9A. Infectivity for p51W401-p66W410 dimer interface

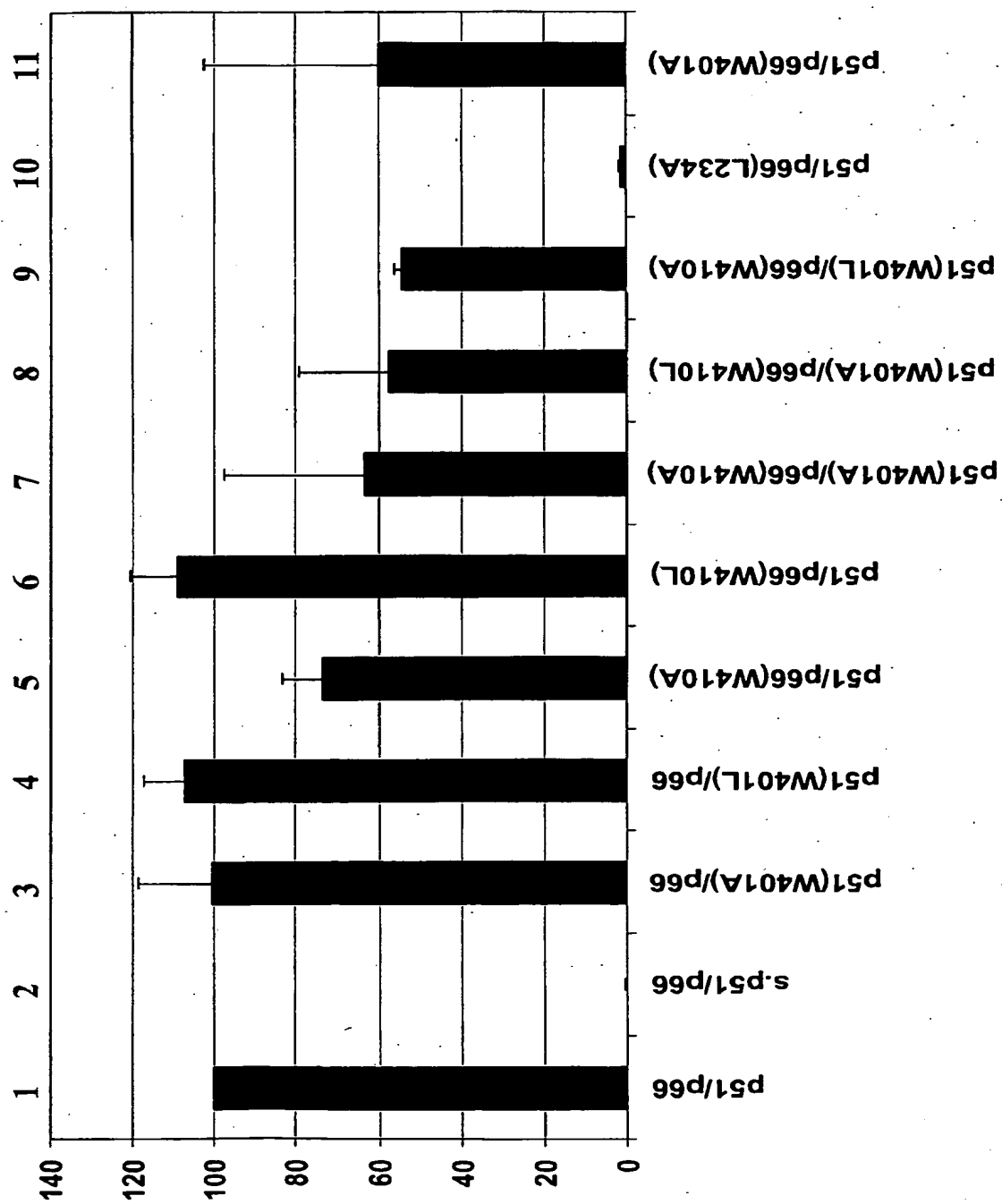
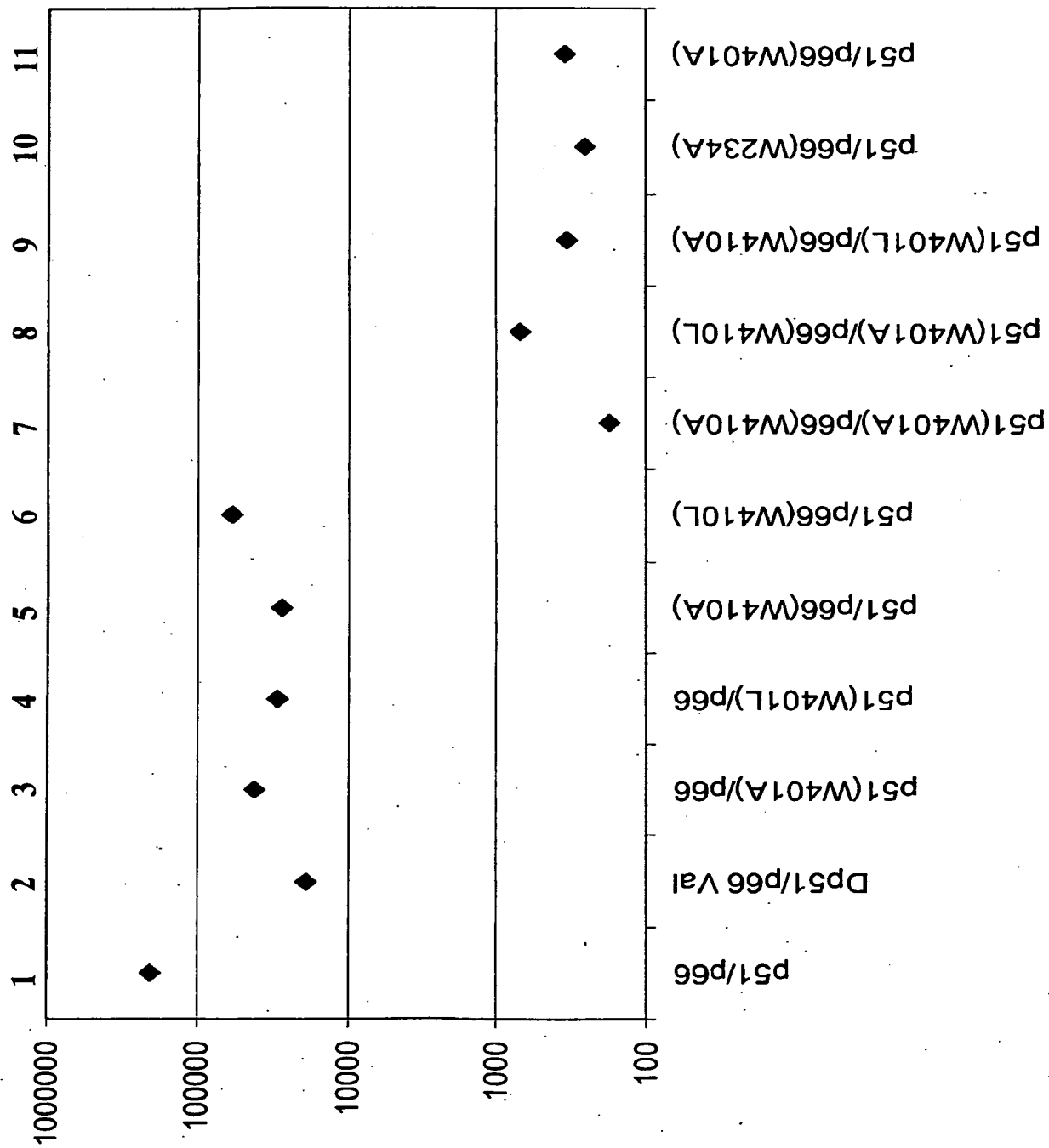


Fig 9B. RT assay for p51W401-p66W410 dimer interface



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    370                375                380
Lys Thr Pro Lys Phe Lys Leu Pro Ile Gln Lys Glu Thr Trp Glu Thr
385                390                395                400
Trp Trp Thr Glu Tyr Trp Gln Ala Thr Trp Ile Pro Glu Trp Glu Phe
    405                410                415
Val Asn Thr Pro Pro Leu Val Lys Leu Trp Tyr Gln Leu Glu Lys Glu
    420                425                430
Pro Ile Val Gly Ala Glu Thr Phe
    435                440

```

<210> 5

<211> 440

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 5

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Pro Ile Ser Pro Ile Glu Thr Val Pro Val Lys Leu Lys Pro Gly Met
  1                5                10                15
Asp Gly Pro Lys Val Lys Gln Trp Pro Leu Thr Glu Glu Lys Ile Lys
    20                25                30
Ala Leu Val Glu Ile Cys Thr Glu Met Glu Lys Glu Gly Lys Ile Ser
    35                40                45
Lys Ile Gly Pro Glu Asn Pro Tyr Asn Thr Pro Val Phe Ala Ile Lys
    50                55                60
Lys Lys Asp Ser Thr Lys Trp Arg Lys Leu Val Asp Phe Arg Glu Leu
65                70                75                80
Asn Lys Arg Thr Gln Asp Phe Trp Glu Val Gln Leu Gly Ile Pro His
    85                90                95
Pro Ala Gly Leu Lys Lys Lys Lys Ser Val Thr Val Leu Asp Val Gly
    100                105                110
Asp Ala Tyr Phe Ser Val Pro Leu Asp Glu Asp Phe Arg Lys Tyr Thr
    115                120                125
Ala Phe Thr Ile Pro Ser Ile Asn Asn Glu Thr Pro Gly Ile Arg Tyr
    130                135                140

```

ATTORNEY DOCKET NO. 21085.0123U1

Gln	Tyr	Asn	Val	Leu	Pro	Gln	Gly	Trp	Lys	Gly	Ser	Pro	Ala	Ile	Phe
145					150					155					160
Gln	Ser	Ser	Met	Thr	Lys	Ile	Leu	Glu	Pro	Phe	Arg	Lys	Gln	Asn	Pro
				165					170						175
Asp	Ile	Val	Ile	Tyr	Gln	Tyr	Met	Asp	Asp	Leu	Tyr	Val	Gly	Ser	Asp
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Leu	Glu	Ile	Gly	Gln	His	Arg	Thr	Lys	Ile	Glu	Glu	Leu	Arg	Gln	His
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Leu	Leu	Arg	Trp	Gly	Leu	Thr	Thr	Pro	Asp	Lys	Lys	His	Gln	Lys	Glu
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Pro	Pro	Phe	Leu	Trp	Met	Gly	Tyr	Glu	Leu	His	Pro	Asp	Lys	Trp	Thr
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Ile	Gln	Lys	Leu	Val	Gly	Lys	Leu	Asn	Trp	Ala	Ser	Gln	Ile	Tyr	Pro
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Gly	Ile	Lys	Val	Arg	Gln	Leu	Cys	Lys	Leu	Leu	Arg	Gly	Thr	Lys	Ala
	275						280					285			
Leu	Thr	Glu	Val	Ile	Pro	Leu	Thr	Glu	Glu	Ala	Glu	Leu	Glu	Leu	Ala
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Glu	Asn	Arg	Glu	Ile	Leu	Lys	Glu	Pro	Val	His	Gly	Val	Tyr	Tyr	Asp
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Lys	Tyr	Ala	Arg	Met	Arg	Gly	Ala	His	Thr	Asn	Asp	Val	Lys	Gln	Leu
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Thr	Glu	Ala	Val	Gln	Lys	Ile	Thr	Thr	Glu	Ser	Ile	Val	Ile	Trp	Gly
	370					375					380				
Lys	Thr	Pro	Lys	Phe	Lys	Leu	Pro	Ile	Gln	Lys	Glu	Thr	Trp	Glu	Thr
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		435					440								

<210> 6

<211> 170

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 6

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1				5					10					15	
Leu	Gly	Lys	Ile	Trp	Pro	Ser	His	Lys	Gly	Arg	Pro	Gly	Asn	Phe	Leu
			20					25					30		
Gln	Ser	Arg	Pro	Glu	Pro	Thr	Ala	Pro	Pro	Glu	Glu	Ser	Phe	Arg	Cys
		35				40						45			
Gly	Glu	Glu	Lys	Thr	Thr	Pro	Pro	Gln	Lys	Pro	Glu	Gln	Thr	Asp	Lys
	50					55				60					
Glu	Leu	Tyr	Pro	Leu	Ala	Ser	Leu	Arg	Ser	Leu	Phe	Gly	Gln	Arg	Pro
65					70					75					80

ATTORNEY DOCKET NO. 21085.0123U1

Leu Val Thr Ile Lys Ile Gly Gly Gln Leu Lys Glu Ala Leu Leu Asp
85 90 95
Thr Gly Ala Asp Asp Thr Val Leu Glu Asp Met Ser Leu Pro Gly Lys
100 105 110
Trp Lys Pro Lys Met Ile Gly Gly Ile Gly Gly Phe Ile Lys Val Arg
115 120 125
Gln Tyr Asp Gln Ile Pro Ile Glu Ile Cys Gly His Lys Ala Ile Gly
130 135 140
Thr Val Leu Ile Gly Pro Thr Pro Val Asn Ile Ile Gly Arg Asn Leu
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<210> 7

<211> 511

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

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cccaccagaa	gagagcttca	ggtgtgggga	ggagaaaaca	actccccctc	agaagccgga	180
gcagacagac	aaggaactgt	atccttttagc	ttccctcaga	tcactctttg	gcaacgaccc	240
ctcgtcacaa	taaagatagg	ggggcagcta	aaggaagctc	tattagatac	aggagcagat	300
gatacagtat	tagaagacat	gagtttgcca	ggaaaaatgga	agccaaaaat	gataggggga	360
attggaggtt	ttatcaaagt	aagacagtat	gatcagatac	ctatagaaat	ctgtgggcat	420
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<210> 8

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

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<210> 9

<211> 4

<212> PRT

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<223> Description of Artificial Sequence; note =
synthetic construct

<221> VARIANT

<222> 2

<223> Xaa = any amino acid

ATTORNEY DOCKET NO. 21085.0123U1

<400> 9

Tyr Xaa Asp Asp

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<210> 10

<211> 26

<212> DNA

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26

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<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence; note =
synthetic construct

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<211> 45

<212> DNA

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<223> Description of Artificial Sequence; note =
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<212> DNA

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tatgtcgaca cccaattatg aaaag

25

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ATTORNEY DOCKET NO. 21085.0123U1

<400> 14
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<400> 15
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<212> DNA
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<400> 16
ttattaacgc gtccgcccct ctccctcccc cc 32

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<210> 18
<211> 21
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<400> 18
agcttgccct gagtgcttca a 21

<210> 19
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synthetic construct

ATTORNEY DOCKET NO. 21085.0123U1

<400> 19
ctgctagaga ttttcacac tgacta 26

<210> 20
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<400> 20
ggctagctag ggaaccact g 21

<210> 21
<211> 22
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<213> Artificial Sequence

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<223> Description of Artificial Sequence; note =
synthetic construct

<400> 21
atactgacgc tctgcaccc at 22

From the INTERNATIONAL BUREAU

PCTNOTIFICATION OF THE RECORDING
OF A CHANGE(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

To:

FISH & RICHARDSON P.C.
P.O. Box 1022
Minneapolis, MN 55440-1022
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 22 September 2006 (22.09.2006)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference 20674-010WO1	
International application No. PCT/US2005/018335	International filing date (day/month/year) 24 May 2005 (24.05.2005)

1. The following indications appeared on record concerning:

☐ the applicant ☐ the inventor ☒ the agent ☐ the common representative

Name and Address MCKEON, Tina, Williams Needle & Rosenberg, P.C. Suite 1000 999 Peachtree Street Atlanta, GA 30309-3915 United States of America	State of Nationality	State of Residence
	Telephone No. 678 420 9300	
	Facsimile No. 678 420 9301	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☐ the person ☐ the name ☐ the address ☐ the nationality ☐ the residence

Name and Address FISH & RICHARDSON P.C. P.O. Box 1022 Minneapolis, MN 55440-1022 United States of America	State of Nationality	State of Residence
	Telephone No. 404 942 2747	
	Facsimile No. 877 769 7945	
	Teleprinter No.	

3. Further observations, if necessary:

The appointment of the agent of record has been revoked. A new agent has been appointed, as indicated in Box 2. The new agent's file reference is : 20674-010WO1

4. A copy of this notification has been sent to:

<input checked="" type="checkbox"/> the receiving Office	<input checked="" type="checkbox"/> the designated Offices concerned
<input checked="" type="checkbox"/> the International Searching Authority	<input type="checkbox"/> the elected Offices concerned
<input type="checkbox"/> the International Preliminary Examining Authority	<input type="checkbox"/> other:

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Campin Helene Facsimile No. +41 22 338 89 65 Telephone No. +41 22 338 97 16
---	---

From the INTERNATIONAL BUREAU

PCTNOTIFICATION CONCERNING
SUBMISSION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) 09 November 2005 (09.11.2005)		To: McKEON, Tina, Williams Needle & Rosenberg, P.C. Suite 1000 999 Peachtree Street Atlanta, GA 30309-3915 ETATS-UNIS D'AMERIQUE
Applicant's or agent's file reference 21085.0123P1		
International application No. PCT/US2005/018335	International filing date (day/month/year) 24 May 2005 (24.05.2005)	
International publication date (day/month/year)	Priority date (day/month/year) 24 May 2004 (24.05.2004)	
Applicant THE UAB RESEARCH FOUNDATION et al		

- By means of this Form, which replaces any previously issued notification concerning submission or transmittal of priority documents, the applicant is hereby notified of the date of receipt by the International Bureau of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to a date of receipt, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
- (If applicable)* The letters "NR" appearing in the right-hand column denote a priority document which, on the date of mailing of this Form, had not yet been received by the International Bureau under Rule 17.1(a) or (b). Where, under Rule 17.1(a), the priority document must be submitted by the applicant to the receiving Office or the International Bureau, but the applicant fails to submit the priority document within the applicable time limit under that Rule, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
- (If applicable)* An asterisk (*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b) (the priority document was received after the time limit prescribed in Rule 17.1(a) or the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17.1(b)). Even though the priority document was not furnished in compliance with Rule 17.1(a) or (b), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17.1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

<u>Priority date</u>	<u>Priority application No.</u>	<u>Country or regional Office or PCT receiving Office</u>	<u>Date of receipt of priority document</u>
24 May 2004 (24.05.2004)	60/573,918	US	05 October 2005 (05.10.2005)
06 April 2005 (06.04.2005)	60/668,858	US	05 October 2005 (05.10.2005)

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Sita COPPOLINO
Facsimile No. +41 22 338 82 70	Facsimile No. (41-22) 338.89.65 Telephone No. +41 22 338 8451

From the INTERNATIONAL BUREAU

PCTNOTIFICATION CONCERNING
SUBMISSION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) 09 November 2005 (09.11.2005)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference 21085.0123P1	
International application No. PCT/US2005/018335	
International publication date (day/month/year)	
Applicant THE UAB RESEARCH FOUNDATION et al	International filing date (day/month/year) 24 May 2005 (24.05.2005)
	Priority date (day/month/year) 24 May 2004 (24.05.2004)

1. By means of this Form, which replaces any previously issued notification concerning submission or transmittal of priority documents, the applicant is hereby notified of the date of receipt by the International Bureau of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to a date of receipt, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
2. (If applicable) The letters "NR" appearing in the right-hand column denote a priority document which, on the date of mailing of this Form, had not yet been received by the International Bureau under Rule 17.1(a) or (b). Where, under Rule 17.1(a), the priority document must be submitted by the applicant to the receiving Office or the International Bureau, but the applicant fails to submit the priority document within the applicable time limit under that Rule, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
3. (If applicable) An asterisk (*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b) (the priority document was received after the time limit prescribed in Rule 17.1(a) or the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17.1(b)). Even though the priority document was not furnished in compliance with Rule 17.1(a) or (b), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17.1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

<u>Priority date</u>	<u>Priority application No.</u>	<u>Country or regional Office or PCT receiving Office</u>	<u>Date of receipt of priority document</u>
06 April 2005 (06.04.2005)	60/668,858	US	05 October 2005 (05.10.2005)

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Sita COPPOLINO Facsimile No. (41-22) 338.89.65 Telephone No. +41 22 338 8451
Facsimile No. +41 22 338 82 70	

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
TIFFANY B. SALMON
FISH & RICHARDSON PC
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Applicant's or agent's file reference 21085.0123P1		Date of mailing (day/month/year) 16 MAY 2007 FOR FURTHER ACTION See paragraph 2 below
International application No. PCT/US05/18335	International filing date (day/month/year) 24 May 2005 (24.05.2005)	Priority date (day/month/year) 24 May 2004 (24.05.2004)
International Patent Classification (IPC) or both national classification and IPC IPC: C12P 1/00(2006.01) USPC: 435/41		
Applicant THE UAB RESEARCH FOUNDATION		

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☒ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

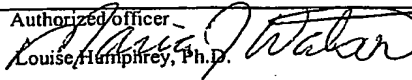
2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA/ US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Date of completion of this opinion 27 April 2007 (27.04.2007)	Authorized officer  Louise Humphrey, Ph.D. Telephone No. 571-272-1600
--	--	--

Form PCT/ISA/237 (cover sheet) (April 2005)

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US05/18335

Box No. 1 Basis of this opinion

1. With regard to the language, this opinion has been established on the basis of:

- ☒ the international application in the language in which it was filed
- ☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b));^a

2. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

a. type of material

- ☐ a sequence listing
- ☐ table(s) related to the sequence listing

b. format of material

- ☐ on paper
- ☐ in electronic form

c. time of filing/furnishing

- ☐ contained in the international application as filed.
- ☐ filed together with the international application in electronic form.
- ☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US05/18335

Box No. IV Lack of unity of invention

1. ☐ In response to the invitation (Form PCT/ISA/206) to pay additional fees the applicant has, within the applicable time limit:
- ☐ paid additional fees
 - ☐ paid additional fees under protest and, where applicable, the protest fee
 - ☐ paid additional fees under protest but the applicable protest fee was not paid
 - ☐ not paid additional fees
2. ☒ This Authority found that the requirement of unity of invention is not complied with and chose not to invite the applicant to pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rule 13.1, 13.2 and 13.3 is
- ☐ complied with
 - ☒ not complied with for the following reasons:
See the lack of unity section of the International Search Report (Form PCT/ISA/210)

4. Consequently, this opinion has been established in respect of the following parts of the international application:

- ☐ all parts.
- ☒ the parts relating to claims Nos. 1, 30, 31 and 34-36

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US05/18335

Box No. V Reasoned statement under Rule 43 bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims <u>30, 31</u>	YES
	Claims <u>1, 34-36</u>	NO
Inventive step (IS)	Claims <u>30, 31</u>	YES
	Claims <u>1, 34-36</u>	NO
Industrial applicability (IA)	Claims <u>1, 30, 31, 34-36</u>	YES
	Claims <u>NONE</u>	NO

2. Citations and explanations:

Claims 1 and 34-36 lack novelty under PCT Article 33(2) as being anticipated by Wu *et al.* (1997). The instant claims are directed to a cell comprising a vector comprising a p66 subunit, a p51 subunit, and Vpr, wherein Vpr and p51 are expressed as a fusion protein; and a retrovirus proviral DNA.

Wu *et al.* teach the pLR2P-vprRT vector that comprising HIV-1 *vpr*, LTR, RRE, and a gene encoding reverse transcriptase, which comprises the p66, p51, and RNase H subunit. The Vpr and RT are expressed as a fusion protein. The pLR2P-vprRT is cotransfected into 293T cells with HIV-1 mutant proviral clones. See page 5114, *Incorporation of Vpr-RT fusion protein into HIV-1 particles*.

Claims 1 and 34-36 lack novelty under PCT Article 33(2) as being anticipated by Shehu-Xhilaga *et al.* (2002). Shehu-Xhilaga *et al.* teach Vpr-RT expression plasmids and the cotransfection of the plasmids into 293T cells with proviral DNA. See page 4332, left column, *DNA plasmids* and *Virus production*.

Claims 30 and 31 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest the expression cassette with the genes arranged in the exact order of LTR-vpr-p51-IRES-p66.

Claims 1, 30, 31 and 34-36 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY (Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44bis)

Applicant's or agent's file reference 20674-010WO1	FOR FURTHER ACTION		See item 4 below
International application No. PCT/US2005/018335	International filing date (<i>day/month/year</i>) 24 May 2005 (24.05.2005)	Priority date (<i>day/month/year</i>) 24 May 2004 (24.05.2004)	
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237			
Applicant THE UAB RESEARCH FOUNDATION			

<p>1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 bis.1(a).</p> <p>2. This REPORT consists of a total of 5 sheets, including this cover sheet.</p> <p>In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.</p>																
<p>3. This report contains indications relating to the following items:</p> <table> <tr> <td><input checked="" type="checkbox"/> Box No. I</td> <td>Basis of the report</td> </tr> <tr> <td><input type="checkbox"/> Box No. II</td> <td>Priority</td> </tr> <tr> <td><input type="checkbox"/> Box No. III</td> <td>Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</td> </tr> <tr> <td><input checked="" type="checkbox"/> Box No. IV</td> <td>Lack of unity of invention</td> </tr> <tr> <td><input checked="" type="checkbox"/> Box No. V</td> <td>Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</td> </tr> <tr> <td><input type="checkbox"/> Box No. VI</td> <td>Certain documents cited</td> </tr> <tr> <td><input type="checkbox"/> Box No. VII</td> <td>Certain defects in the international application</td> </tr> <tr> <td><input type="checkbox"/> Box No. VIII</td> <td>Certain observations on the international application</td> </tr> </table> <p>4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44bis.3(c) and 93bis.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44bis .2).</p>	<input checked="" type="checkbox"/> Box No. I	Basis of the report	<input type="checkbox"/> Box No. II	Priority	<input type="checkbox"/> Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability	<input checked="" type="checkbox"/> Box No. IV	Lack of unity of invention	<input checked="" type="checkbox"/> Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement	<input type="checkbox"/> Box No. VI	Certain documents cited	<input type="checkbox"/> Box No. VII	Certain defects in the international application	<input type="checkbox"/> Box No. VIII	Certain observations on the international application
<input checked="" type="checkbox"/> Box No. I	Basis of the report															
<input type="checkbox"/> Box No. II	Priority															
<input type="checkbox"/> Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability															
<input checked="" type="checkbox"/> Box No. IV	Lack of unity of invention															
<input checked="" type="checkbox"/> Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement															
<input type="checkbox"/> Box No. VI	Certain documents cited															
<input type="checkbox"/> Box No. VII	Certain defects in the international application															
<input type="checkbox"/> Box No. VIII	Certain observations on the international application															

<p>The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland</p> <p>Facsimile No. +41 22 338 82 70</p>	Date of issuance of this report 19 June 2007 (19.06.2007)
	<p>Authorized officer</p> <p>Nora Lindner</p> <p>e-mail: pt02.pct@wipo.int</p>

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
TIFFANY B. SALMON
FISH & RICHARDSON PC
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Applicant's or agent's file reference 21085.0123P1		Date of mailing (day/month/year) 16 MAY 2007
International application No. PCT/US05/18335		International filing date (day/month/year) 24 May 2005 (24.05.2005)
International Patent Classification (IPC) or both national classification and IPC IPC: C12P 1/00(2006.01) USPC: 435/41		Priority date (day/month/year) 24 May 2004 (24.05.2004)
Applicant THE UAB RESEARCH FOUNDATION		

1. This opinion contains indications relating to the following items:

- | | | |
|-------------------------------------|--------------|--|
| <input checked="" type="checkbox"/> | Box No. I | Basis of the opinion |
| <input type="checkbox"/> | Box No. II | Priority |
| <input type="checkbox"/> | Box No. III | Non-establishment of opinion with regard to novelty, inventive step and industrial applicability |
| <input checked="" type="checkbox"/> | Box No. IV | Lack of unity of invention |
| <input checked="" type="checkbox"/> | Box No. V | Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement |
| <input type="checkbox"/> | Box No. VI | Certain documents cited |
| <input type="checkbox"/> | Box No. VII | Certain defects in the international application |
| <input type="checkbox"/> | Box No. VIII | Certain observations on the international application |

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA/ US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Date of completion of this opinion 27 April 2007 (27.04.2007)	Authorized officer Louise Humphrey, Ph.D. Telephone No. 571-272-1600
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Form PCT/ISA/237 (cover sheet) (April 2005)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US05/18335

Box No. I Basis of this opinion

1. With regard to the language, this opinion has been established on the basis of:

- ☒ the international application in the language in which it was filed
- ☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).*

2. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

a. type of material

- ☐ a sequence listing
- ☐ table(s) related to the sequence listing

b. format of material

- ☐ on paper
- ☐ in electronic form

c. time of filing/furnishing

- ☐ contained in the international application as filed.
- ☐ filed together with the international application in electronic form.
- ☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US05/18335

Box No. IV Lack of unity of invention

1. ☐ In response to the invitation (Form PCT/ISA/206) to pay additional fees the applicant has, within the applicable time limit:
- ☐ paid additional fees
 - ☐ paid additional fees under protest and, where applicable, the protest fee
 - ☐ paid additional fees under protest but the applicable protest fee was not paid
 - ☐ not paid additional fees
2. ☒ This Authority found that the requirement of unity of invention is not complied with and chose not to invite the applicant to pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rule 13.1, 13.2 and 13.3 is
- ☐ complied with
 - ☒ not complied with for the following reasons:
See the lack of unity section of the International Search Report (Form PCT/ISA/210)

4. Consequently, this opinion has been established in respect of the following parts of the international application:
- ☐ all parts.
 - ☒ the parts relating to claims Nos. 1, 30, 31 and 34-36

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US05/18335

Box No. V Reasoned statement under Rule 43 bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims <u>30, 31</u>	YES
	Claims <u>1, 34-36</u>	NO
Inventive step (IS)	Claims <u>30, 31</u>	YES
	Claims <u>1, 34-36</u>	NO
Industrial applicability (IA)	Claims <u>1, 30, 31, 34-36</u>	YES
	Claims <u>NONE</u>	NO

2. Citations and explanations:

Claims 1 and 34-36 lack novelty under PCT Article 33(2) as being anticipated by Wu *et al.* (1997).
The instant claims are directed to a cell comprising a vector comprising a p66 subunit, a p51 subunit, and Vpr, wherein Vpr and p51 are expressed as a fusion protein; and a retrovirus proviral DNA.

Wu *et al.* teach the pLR2P-vprRT vector that comprising HIV-1 *vpr*, LTR, RRE, and a gene encoding reverse transcriptase, which comprises the p66, p51, and RNase H subunit. The Vpr and RT are expressed as a fusion protein. The pLR2P-vprRT is cotransfected into 293T cells with HIV-1 mutant proviral clones. See page 5114, *Incorporation of Vpr-RT fusion protein into HIV-1 particles*.

Claims 1 and 34-36 lack novelty under PCT Article 33(2) as being anticipated by Shehu-Xhilaga *et al.* (2002).
Shehu-Xhilaga *et al.* teach Vpr-RT expression plasmids and the cotransfection of the plasmids into 293T cells with proviral DNA. See page 4332, left column, *DNA plasmids* and *Virus production*.

Claims 30 and 31 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest the expression cassette with the genes arranged in the exact order of LTR-vpr-p51-IRES-p66.

Claims 1, 30, 31 and 34-36 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.

SEQUENCE LISTING

<110> UAB Research Foundation

KAPPES, John C.
 MULKY, Alok
 WU, Xiaoyun

<120> METHODS AND COMPOSITIONS FOR IDENTIFYING
 COMPOUNDS THAT INHIBIT HIV-1 SUBUNIT-SPECIFIC REVERSE
 TRANSCRIPTASE

<130> 21085.0123P1

<140> Unassigned

<141> 2005-05-24

<150> 60/573,918

<151> 2004-05-24

<150> 60/668,858

<151> 2005-04-06

<160> 21

<170> FastSEQ for Windows Version 4.0

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<211> 858

<212> DNA

<213> Artificial Sequence

<220>

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acgagtggac	cctggagctg	ctggaggagc	tgaagaggga	ggccgtgagg	cacttccccca	120
ggccctggct	gcacggcctg	ggccagcaca	tctacgagac	ctacggcgac	acctggggccg	180
gcgtggaggc	catcatcagg	atcctgcagc	agctgctgtt	catccacttc	aggatcggct	240
gccagcacag	caggatcggc	atcatccagc	agaggagggc	caggaggaac	ggcgccagca	300
ggagctagtt	taaacactgc	acagagagac	aggctaattt	tttagggaaa	atttggcctt	360
ccaacaaagg	gaggccaggg	aattttctcc	agaacaggcc	agagccaaca	gccccacccg	420
cagagagcct	cgggttcgga	gaggagatag	ccccctcccc	gaaacaagag	ccgaaggaaa	480
aggagttata	cccttaacc	tcctcaaat	cactctttgg	cagcgacccc	tagtcacagt	540
aagaataggg	ggacagctaa	tagaagccct	gttagacaca	ggagcagatg	atacagtgtt	600
agaagatata	aatttaccag	gaaaatggaa	acaaaaaatg	atagggggaa	ttggtggtct	660
tatcaaagta	agacagtatg	atcaaatact	tatagaaatt	tgtggaaaaa	aggctatagg	720
gacagtatta	gtaggaccta	cacctatcaa	cataattggg	agaaatatgt	tgactcagat	780
tggttggtact	ttaaattttc	caattagtcc	tattgaaact	gtaccagtaa	aattaaagcc	840
aggaatggat	ggtccaaa					858

<210> 2

<211> 96

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

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Met Glu Gln Ala Pro Glu Asp Gln Gly Pro Pro Arg Glu Pro Tyr Asn
 1           5           10           15
Ala Trp Thr Leu Glu Leu Leu Glu Glu Leu Lys Ser Glu Ala Val Arg
          20           25           30
His Phe Pro Arg Val Trp Leu His Gly Leu Gly Gln His Ile Tyr Glu
          35           40           45
Thr Tyr Gly Asp Thr Trp Ala Gly Val Glu Ala Ile Ile Arg Ile Leu
          50           55           60
Gln Gln Leu Leu Phe Ile His Phe Arg Ile Gly Cys Gln His Ser Arg
65           70           75           80
Ile Gly Ile Thr Arg Gln Arg Arg Ala Arg Asn Gly Ala Ser Arg Ser
          85           90           95

```

<210> 3

<211> 315

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 3

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gtttaaacgc caccatggag caggcccccg aggaccaggg cccccagagg gagccccaca      60
acgagtggac cctggagctg ctggaggagc tgaagagggg ggccgtgagg cacttcccca      120
ggccctggct gcacggcctg ggccagcaca tctacgagac ctacggcgac acctgggccc      180
gcgtggaggc catcatcagg atcctgcagc agctgctgtt catccacttc aggatcggct      240
gccagcacag caggatcggc atcatccagc agaggagggc caggaggaac ggcgccagca      300
ggagctagtt taaac                                     315

```

<210> 4

<211> 440

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 4

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Pro Ile Ser Pro Ile Glu Thr Val Pro Val Lys Leu Lys Pro Gly Met
 1           5           10           15
Asp Gly Pro Lys Val Lys Gln Trp Pro Leu Thr Glu Glu Lys Ile Lys
          20           25           30
Ala Leu Val Glu Ile Cys Thr Glu Met Glu Lys Glu Gly Lys Ile Ser
          35           40           45
Lys Ile Gly Pro Glu Asn Pro Tyr Asn Thr Pro Val Phe Ala Ile Lys
          50           55           60
Lys Lys Asp Ser Thr Lys Trp Arg Lys Leu Val Asp Phe Arg Glu Leu
65           70           75           80
Asn Lys Arg Thr Gln Asp Phe Trp Glu Val Gln Leu Gly Ile Pro His
          85           90           95
Pro Ala Gly Leu Lys Lys Lys Lys Ser Val Thr Val Leu Asp Val Gly
          100          105          110
Asp Ala Tyr Phe Ser Val Pro Leu Asp Glu Asp Phe Arg Lys Tyr Thr
          115          120          125

```

Ala Phe Thr Ile Pro Ser Ile Asn Asn Glu Thr Pro Gly Ile Arg Tyr
 130 135 140
 Gln Tyr Asn Val Leu Pro Gln Gly Trp Lys Gly Ser Pro Ala Ile Phe
 145 150 155 160
 Gln Ser Ser Met Thr Lys Ile Leu Glu Pro Phe Arg Lys Gln Asn Pro
 165 170 175
 Asp Ile Val Ile Tyr Gln Tyr Met Asp Asp Leu Tyr Val Gly Ser Asp
 180 185 190
 Leu Glu Ile Gly Gln His Arg Thr Lys Ile Glu Glu Leu Arg Gln His
 195 200 205
 Leu Leu Arg Trp Gly Leu Thr Thr Pro Asp Lys Lys His Gln Lys Glu
 210 215 220
 Pro Pro Phe Leu Trp Met Gly Tyr Glu Leu His Pro Asp Lys Trp Thr
 225 230 235 240
 Val Gln Pro Ile Val Leu Pro Glu Lys Asp Ser Trp Thr Val Asn Asp
 245 250 255
 Ile Gln Lys Leu Val Gly Lys Leu Asn Trp Ala Ser Gln Ile Tyr Pro
 260 265 270
 Gly Ile Lys Val Arg Gln Leu Cys Lys Leu Leu Arg Gly Thr Lys Ala
 275 280 285
 Leu Thr Glu Val Ile Pro Leu Thr Glu Glu Ala Glu Leu Glu Leu Ala
 290 295 300
 Glu Asn Arg Glu Ile Leu Lys Glu Pro Val His Gly Val Tyr Tyr Asp
 305 310 315 320
 Pro Ser Lys Asp Leu Ile Ala Glu Ile Gln Lys Gln Gly Gln Gly Gln
 325 330 335
 Trp Thr Tyr Gln Ile Tyr Gln Glu Pro Phe Lys Asn Leu Lys Thr Gly
 340 345 350
 Lys Tyr Ala Arg Met Arg Gly Ala His Thr Asn Asp Val Lys Gln Leu
 355 360 365
 Thr Glu Ala Val Gln Lys Ile Thr Thr Glu Ser Ile Val Ile Trp Gly
 370 375 380
 Lys Thr Pro Lys Phe Lys Leu Pro Ile Gln Lys Glu Thr Trp Glu Thr
 385 390 395 400
 Trp Trp Thr Glu Tyr Trp Gln Ala Thr Trp Ile Pro Glu Trp Glu Phe
 405 410 415
 Val Asn Thr Pro Pro Leu Val Lys Leu Trp Tyr Gln Leu Glu Lys Glu
 420 425 430
 Pro Ile Val Gly Ala Glu Thr Phe
 435 440

<210> 5

<211> 440

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 5

Pro Ile Ser Pro Ile Glu Thr Val Pro Val Lys Leu Lys Pro Gly Met
 1 5 10 15
 Asp Gly Pro Lys Val Lys Gln Trp Pro Leu Thr Glu Glu Lys Ile Lys
 20 25 30
 Ala Leu Val Glu Ile Cys Thr Glu Met Glu Lys Glu Gly Lys Ile Ser
 35 40 45
 Lys Ile Gly Pro Glu Asn Pro Tyr Asn Thr Pro Val Phe Ala Ile Lys
 50 55 60
 Lys Lys Asp Ser Thr Lys Trp Arg Lys Leu Val Asp Phe Arg Glu Leu
 65 70 75 80

```

Asn Lys Arg Thr Gln Asp Phe Trp Glu Val Gln Leu Gly Ile Pro His
      85                      90                      95
Pro Ala Gly Leu Lys Lys Lys Lys Ser Val Thr Val Leu Asp Val Gly
      100                    105                    110
Asp Ala Tyr Phe Ser Val Pro Leu Asp Glu Asp Phe Arg Lys Tyr Thr
      115                    120                    125
Ala Phe Thr Ile Pro Ser Ile Asn Asn Glu Thr Pro Gly Ile Arg Tyr
      130                    135                    140
Gln Tyr Asn Val Leu Pro Gln Gly Trp Lys Gly Ser Pro Ala Ile Phe
      145                    150                    155                    160
Gln Ser Ser Met Thr Lys Ile Leu Glu Pro Phe Arg Lys Gln Asn Pro
      165                    170                    175
Asp Ile Val Ile Tyr Gln Tyr Met Asp Asp Leu Tyr Val Gly Ser Asp
      180                    185                    190
Leu Glu Ile Gly Gln His Arg Thr Lys Ile Glu Glu Leu Arg Gln His
      195                    200                    205
Leu Leu Arg Trp Gly Leu Thr Thr Pro Asp Lys Lys His Gln Lys Glu
      210                    215                    220
Pro Pro Phe Leu Trp Met Gly Tyr Glu Leu His Pro Asp Lys Trp Thr
      225                    230                    235                    240
Val Gln Pro Ile Val Leu Pro Glu Lys Asp Ser Trp Thr Val Asn Asp
      245                    250                    255
Ile Gln Lys Leu Val Gly Lys Leu Asn Trp Ala Ser Gln Ile Tyr Pro
      260                    265                    270
Gly Ile Lys Val Arg Gln Leu Cys Lys Leu Leu Arg Gly Thr Lys Ala
      275                    280                    285
Leu Thr Glu Val Ile Pro Leu Thr Glu Glu Ala Glu Leu Glu Leu Ala
      290                    295                    300
Glu Asn Arg Glu Ile Leu Lys Glu Pro Val His Gly Val Tyr Tyr Asp
      305                    310                    315                    320
Pro Ser Lys Asp Leu Ile Ala Glu Ile Gln Lys Gln Gly Gln Gly Gln
      325                    330                    335
Trp Thr Tyr Gln Ile Tyr Gln Glu Pro Phe Lys Asn Leu Lys Thr Gly
      340                    345                    350
Lys Tyr Ala Arg Met Arg Gly Ala His Thr Asn Asp Val Lys Gln Leu
      355                    360                    365
Thr Glu Ala Val Gln Lys Ile Thr Thr Glu Ser Ile Val Ile Trp Gly
      370                    375                    380
Lys Thr Pro Lys Phe Lys Leu Pro Ile Gln Lys Glu Thr Trp Glu Thr
      385                    390                    395                    400
Trp Trp Thr Glu Tyr Trp Gln Ala Thr Trp Ile Pro Glu Trp Glu Phe
      405                    410                    415
Val Asn Thr Pro Pro Leu Val Lys Leu Trp Tyr Gln Leu Glu Lys Glu
      420                    425                    430
Pro Ile Val Gly Ala Glu Thr Phe
      435                    440

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<210> 6

<211> 170

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence; note =
synthetic construct

<400> 6

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Lys Glu Gly His Gln Met Lys Glu Cys Thr Glu Arg Gln Ala Asn Phe
  1                      5                      10                      15
Leu Gly Lys Ile Trp Pro Ser His Lys Gly Arg Pro Gly Asn Phe Leu
      20                      25                      30

```

Gln Ser Arg Pro Glu Pro Thr Ala Pro Pro Glu Glu Ser Phe Arg Cys
 35 40 45
 Gly Glu Glu Lys Thr Thr Pro Pro Gln Lys Pro Glu Gln Thr Asp Lys
 50 55 60
 Glu Leu Tyr Pro Leu Ala Ser Leu Arg Ser Leu Phe Gly Gln Arg Pro
 65 70 75 80
 Leu Val Thr Ile Lys Ile Gly Gly Gln Leu Lys Glu Ala Leu Leu Asp
 85 90 95
 Thr Gly Ala Asp Asp Thr Val Leu Glu Asp Met Ser Leu Pro Gly Lys
 100 105 110
 Trp Lys Pro Lys Met Ile Gly Gly Ile Gly Gly Phe Ile Lys Val Arg
 115 120 125
 Gln Tyr Asp Gln Ile Pro Ile Glu Ile Cys Gly His Lys Ala Ile Gly
 130 135 140
 Thr Val Leu Ile Gly Pro Thr Pro Val Asn Ile Ile Gly Arg Asn Leu
 145 150 155 160
 Leu Thr Gln Ile Gly Cys Thr Leu Asn Phe
 165 170

<210> 7

<211> 511

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 7

aaaggaagga	caccaaata	aagaatgcac	tgagagacag	gctaattttt	tagggaaaat	60
ctggccttcc	cacaaggga	ggccagggaa	ctttctccag	agcagaccag	agccaacagc	120
cccaccagaa	gagagcttca	ggtgtgggga	ggagaaaaca	actccccctc	agaagccgga	180
gcagacagac	aaggaactgt	atccttttagc	ttccctcaga	tcactctttg	gcaacgaccc	240
ctcgtcacia	taaagatagg	ggggcagcta	aaggaagctc	tattagatac	aggagcagat	300
gatacagtat	tagaagacat	gagtttgcca	ggaaaatgga	agccaaaaat	gataggggga	360
attggagggt	ttatcaaagt	aagacagtat	gatcagatac	ctatagaaat	ctgtgggcat	420
aaagctatag	gtacagtatt	aataggacca	acacctgtca	acataattgg	aagaaatctg	480
ttgacacaga	ttggttcac	tttaaatttt	c			511

<210> 8

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 8

Tyr Met Asp Asp

1

<210> 9

<211> 4

<212> PRT

<213> Artificial Sequence

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<220>
<221> VARIANT
<222> 2
<223> Xaa = any amino acid

<400> 9
Tyr Xaa Asp Asp
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<210> 10
<211> 26
<212> DNA
<213> Artificial Sequence

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<400> 10
aagcccggga tggatggccc aaaagt

26

<210> 11
<211> 45
<212> DNA
<213> Artificial Sequence

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<400> 11
tcctaaacgc gtctccctct aagctgctca atttacttag aaagt

45

<210> 12
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
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<400> 12
actttctaag taaattgagc agcttagagg gagacgcgtt tagga

45

<210> 13
<211> 25
<212> DNA
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<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 13
tatgtcgaca cccaattatg aaaag

25

<210> 14
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<213> Artificial Sequence

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<223> Description of Artificial Sequence; note =
synthetic construct

<400> 14
tagatcagat ctgttgactc agattgggtg ca 32

<210> 15
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<212> DNA
<213> Artificial Sequence

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synthetic construct

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atctacacgc'gtttagaagg tttctgcgcc tt 32

<210> 16
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<213> Artificial Sequence

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ttattaacgc gtccgcccct ctccctcccc cc 32

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tatacgtctt 69

<210> 18
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<223> Description of Artificial Sequence; note =
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<400> 18
agcttgccctt gagtgcttca a 21

<210> 19
<211> 26
<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence; note =
synthetic construct

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ctgctagaga ttttccacac tgacta

26

<210> 20

<211> 21

<212> DNA

<213> Artificial Sequence

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ggctagctag ggaaccact g

21

<210> 21

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 21

atactgacgc tctcgacccc at

22

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
22 December 2005 (22.12.2005)

PCT

(10) International Publication Number
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(51) International Patent Classification:
C12P 1/00 (2006.01)

(21) International Application Number:
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(22) International Filing Date: 24 May 2005 (24.05.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
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(54) Title: METHODS AND COMPOSITIONS FOR IDENTIFYING COMPOUNDS THAT INHIBIT HIV-1 SUBUNIT-SPE-
CIFIC REVERSE TRANSCRIPTASE

(57) Abstract: This invention relates to methods and compositions for identifying compounds that inhibit HIV-1 subunit-specific
reverse transcriptase.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US05/18335

A. CLASSIFICATION OF SUBJECT MATTER

IPC: C12P 1/00(2006.01)

USPC: 435/41

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/41

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WU et al. Functional RT and IN incorporated into HIV-1 particles The EMBO Journal 1997, Vol 16. No. 16, pages 5113-5122, especially page 5114 and 5115.	1, 34-36
X	SHEHU-XHILAGA et al. The conformation of the mature dimeric human immunodeficiency virus type 1 RNA genome requires packaging of Pol protein. Journal of Virology 2002, Vol 76. No. 9, pages 4331-4340, especially page 4332.	1, 34-36
A	PADOW et al. Replication of Chimeric Human Immunodeficiency Virus Type 1 Containing HIV-2 integrase. Journal of Virology, October 2003 Vol 77. No. 20, pages 11050-11059.	1, 30, 31, 34-36



Further documents are listed in the continuation of Box C.



See patent family annex.

Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US05/18335

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of Item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
 2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fees.
 3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

 4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1,30,31 and 34-36
- Remark on Protest**
- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

BOX III. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1, 30, 31, and 34-36, drawn to the technical feature of a cell comprising a vector comprising vpr-p51/66 and an expression cassette comprising LTR-vpr-p51-IRES-p66.

Group II, claims 2-17 and 32, drawn to the technical feature of a method of screening for a compound that inhibits viral reverse transcriptase or affects dimerization of the p66 and a p51 subunit polypeptide of HIV reverse transcriptase.

Group III, claim 18, drawn to the technical feature of a method of making a pharmaceutical composition.

Group IV, claims 19-23, drawn to the technical feature of a method of inhibiting viral reverse transcriptase, inhibiting or enhancing dimerization of the p51 and a p66 subunit polypeptide of HIV-1 reverse transcriptase.

Group V, claims 24 and 27-29, drawn to the technical feature of a compound that inhibits viral reverse transcriptase.

Group VI, claim 25, drawn to the technical feature of a compound that enhances dimerization of the two subunits of HIV-1 reverse transcriptase, p51 and p66.

Group VII, claim 26, drawn to the technical feature of a compound that inhibits dimerization of the two subunits of HIV-1 reverse transcriptase, p51 and p66.

Group VIII, claim 33, drawn to the technical feature of a transgenic animal expressing vpr-p51/66.

The inventions listed as Groups I-VIII do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

As set forth above, each Group requires a technical feature that is not required by any of the other groups.

According to PCT Rule 13.2, unity of invention exists only when the shared same or corresponding special technical feature is a contribution over the prior art.

The technical feature of Group I is a cell containing a vector vpr-p51/66, which is shown by Wu *et al.* (The EMBO journal. 1997, Vol. 16 (16), pp. 5113-5122) to lack novelty as this reference teaches the expression vector of Vpr-RT fusion protein, vpr-p51/p66 (Figure 1B), ligated in-frame and placed under control of LTR and RRE, and 293T cells transfected with the vector (Materials and Methods), thus does not make a contribution over the prior art.

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(57) Abstract: This invention relates to methods and compositions for identifying compounds that inhibit HIV-1 subunit-specific
reverse transcriptase.

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METHODS AND COMPOSITIONS FOR IDENTIFYING COMPOUNDS THAT INHIBIT HIV-1 SUBUNIT-SPECIFIC REVERSE TRANSCRIPTASE

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims priority to U.S. provisional applications, Serial No. 60/573,918 filed on May 24, 2004 and Serial No. 60/668,858, filed April 6, 2005, which are herein incorporated by reference in their entirety

This invention was funded by the National Institutes of Health, Grant No. AI47714. Therefore, the United States Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

BACKGROUND ART

10 The HIV type 1 (HIV-1) reverse transcriptase (RT) is required for the conversion of genomic RNA into double-stranded proviral DNA, catalyzed by the RNA-dependent DNA polymerase and ribonuclease H activities of the enzyme. HIV-1 RT is an asymmetric dimer
15 formed by the association of p66 and p51 polypeptides, which are cleaved from a large Pr¹⁶⁰GagPol precursor by the viral protease during virion assembly. p51 contains identical N-terminal sequences as p66, but lacks the C-terminal ribonuclease H (RNase H) domain (di Marzo et al. Science 231, 1289-1291, 1986). The structure of HIV-1 RT has been elucidated by x-ray crystallography in a variety of configurations, including unliganded
20 (Rodgers et al. Proc. Natl. Acad. Sci. USA 92, 1222-1226, 1995), complexed to nonnucleoside RT inhibitors (Ren, et al. Nat. Struct. Biol. 2, 293-302, 1995), or complexed with double-stranded DNA either with (Huang et al. Science 282, 1669-1675, 1998) or without deoxynucleotide triphosphate (Jacobo-Molina et al. Proc. Natl. Acad. Sci. USA 90, 6320-6324, 1993; Kohlstaedt et al. Science 256, 1783-1790, 1992). Such analyses have
25 shown that p66 can be divided structurally into the polymerase and RNase H domains, with the polymerase domain further divided into the fingers, palm, thumb and connections subdomains. Although p51 has the same polymerase domains as p66, the relative orientations of these individual domains differ markedly, resulting in p51 assuming a closed structure.

30 The RT heterodimer represents the biologically relevant form of the enzyme; the monomeric subunits have only low catalytic activity (Restle, et al. J. Biol. Chem. 265, 8986-8988, 1990). Structural analysis reveals three major contacts between p66 and p51, with

most of the interaction surfaces being largely hydrophobic (Becerra et al Biochemistry 30, 11707-11719, 1991; Wang et al. Proc. Natl. Acad. Sci. USA 91, 7242-7246, 1994). The three contacts comprise an extensive dimer interface that includes the fingers subdomain of p51 with the palm of p66, the connection subdomains of both subunits, and the thumb subdomain of p51 with the RNase H domain of p66.

Several single amino acid substitutions in HIV-1 RT have been shown to inhibit heterodimer association (Ghosh et al. Biochemistry 35, 8553-8562 1996; Wohrl et al. J. Biol. Chem. 272, 17581-17587, 1997; Goel et al. Biochemistry 32, 13012-13018, 1993). These include the mutations L234A, G231A and W229A, all located in the primer grip region of the p66 subunit, and L289K in the thumb subdomain. These mutations are not located at the dimer interface and probably mediate their effects indirectly through conformational changes in the p66 subunit.

Several biochemical assays have been used previously to specifically measure RT dimerization. Some are based on the physical separation of monomers and dimers as determined by analytical ultracentrifugation and gel filtration. Other assays include intrinsic tryptophan fluorescence (Divita et al. FEBS Lett. 324, 153-158, 1993), chemical crosslinking (Debyser et al. Protein Sci. 5, 278-286, 1996), the use of affinity tags (Jacques et al J. Biol. Chem. 269, 1388-1393, 1994) and polymerase activity itself. Although these methods detect dimerization, they either lack specificity or are not easy to perform. Moreover, these assays do not facilitate the rapid genetic analysis of protein-protein interactions under physiological conditions nor are they suitable for high throughput screening for RT dimerization inhibitors.

Understanding the role of the individual RT subunits in RNA-dependent DNA synthesis has been the focus of several studies. These used *in vitro* biochemical methods to analyze the enzymatic activity of purified recombinant HIV-1 RT heterodimers wherein either the p51 or p66 subunit was selectively mutated (Boyer et al., 1994; Hostomsky et al., 1992; Le Grice et al., 1991). There remains a need in the art, however, for *in vivo* methods and compositions for identifying compounds that inhibit HIV-1 subunit-specific reverse transcriptase.

SUMMARY OF THE INVENTION

In accordance with the purpose(s) of this invention, as embodied and broadly described herein, this invention, in one aspect, relates to a cell comprising a vector wherein the vector expresses a fusion protein comprising a p66 subunit, a p51 subunit, and Vpr, and
5 a reverse transcriptase deficient proviral DNA.

In another aspect, the invention relates to a method of screening for a compound that inhibits viral reverse transcriptase.

This invention also relates to a method of screening for a compound that inhibits or enhances dimerization of a p66 subunit polypeptide of reverse transcriptase and a p51
10 subunit polypeptide of reverse transcriptase.

In another aspect, this invention relates to a method of making a pharmaceutical composition and compounds identified by the methods described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this
15 specification, illustrate embodiments of the invention and together with the description, serve to explain the principles of the invention.

Figure 1 shows construction of the *vpr-p51/p66* and FN expression plasmids. (A) Illustration of the *vpr-p51/p66* expression plasmid. The *vpr-p51/p66* expression plasmid was constructed to allow independent expression and subunit-specific analysis of p51 and
20 p66. The *vpr* and p51 coding sequences were fused in-frame, while preserving the N-terminal protease cleavage (PC) site of RT by including 33 base-pairs of contiguous PR sequence 5' of RT. A translational stop codon (TAA) was introduced to terminate RT expression at amino acids 440, which represents the full-length p51 subunit. *vpr-p51* was succeeded by an internal ribosome entry site (IRES). The p66 coding region was inserted 3'
25 of the IRES and was modified to encode Met-Gly on the N-terminus. The *vpr-p51/p66* expression plasmid was used to construct various p51/p66 mutants. Unless otherwise indicated, this was accomplished by inserting p51 or p66 DNA fragments at the *BglIII-MluI* or *XmaI-XhoI* sites, respectively. (B) Illustration of the FN proviral construct. This proviral construct was made from the wild-type pSG3 plasmid using a previously described strategy
30 (Dubay et al., 1992). The clone contains a 110 amino acid deletion (nucleotides 3374-3704)

in the RT reading frame. Most of the RNase H domain and 13 amino acids of the carboxyl end of the polymerase domain were removed, leaving the IN coding region in-frame.

Figure 2 shows a model for *trans* expression and packaging of heterodimeric RT. Cells are cotransfected with HIV-1 and the vpr-p51/p66 expression plasmids. Vpr-p51
5 incorporates p66 through interaction and stable association of the two subunits (Vpr-p51 and p66) within the cellular cytoplasm. Specific interaction between Vpr and Pr55^{Gag} leads to the incorporation of the Vpr-p51/p66 complex into progeny virions. Subsequent cleavage by the viral PR generates wild-type RT heterodimer (p51/p66).

Figure 3 shows virion incorporation and proteolytic processing of *trans*-
10 heterodimeric RT. The FN proviral DNA was transfected alone or cotransfected with either the vpr-p66, vpr-p51/p66, or vprΔp51/p66 expression plasmids. Included as controls were the wild-type SG3 and the RT-IN minus SG3^{S-RT} proviruses. The transfection-derived virions were concentrated by ultracentrifugation, lysed and analyzed by immunoblotting using (A) anti-RT (α-RT), (B) anti-p66 (α-p66) or (C) anti-Gag (α-CA) antibodies.

Figure 4 shows complementation of the M7 provirus eliminates non-Vpr-p51-mediated p66 incorporation. (A) Construction of the M7 proviral plasmid. The M7 construct was derived from the S-RT construct (Wu et al., 1997), which contains a TAA stop codon at the first amino acid positions of RT and IN. In addition to these mutations, M7 has a -1
15 frameshift at amino acid position 14 of RT, three stop codons, 441 (TAA), 444 (TGA) and 447 (TAG), in the RNase H domain and a RNase H catalytic site mutation at 443 (D443N).
20 (B to D) Analysis of virion incorporation and proteolytic processing of Vpr-p51/p66. The M7 proviral DNA was transfected alone or together with the vpr-IN expression plasmid and either the vpr-p66, vpr-p51/p66, or vprΔp51/p66 expression plasmids. The wild-type SG3 was included as a control. Transfection-derived virions were concentrated by
25 ultracentrifugation, lysed and analyzed by immunoblotting using (B) anti-RT (α-RT), (C) anti-p66 (α-p66) or (D) anti-Gag (α-CA) antibodies.

Figure 5 shows infectivity of *trans*-heterodimeric complemented virions. Viruses were derived by transfection of 293T cells as described in Fig. 4 and analyzed for HIV-1 p24-ag concentration. Virus infectivity was analyzed using the TZM-bl reporter cell line as
30 described in Example 1. Infectivity is expressed as a percentage of the wild-type virus control. The results of three independent experiments are shown.

Figure 6 shows subunit-specific analysis of the YMDD motif. The wild-type, control and mutated vpr-p51/expression plasmids, respectively, were cotransfected into 293T cells with the M7 and vpr-IN DNAs. Transfection derived viruses were analyzed for HIV-1 p24-ag concentration. (A) Analysis of infectivity. Infectivity was analyzed from three
5 independent experiments. (B & C) Analysis of viral DNA synthesis. The DNA products of reverse transcription were analyzed as described in Example 1. (B) Early (R-U5) and (C) late (R-gag) products of reverse transcription were amplified from each DNA extract by PCR, resolved on 1.5% agarose gels and stained with ethidium bromide. To approximate the relative amount of each of the amplified DNA products, 10-fold serial dilutions of pSG3
10 DNA (ranging from 10^1 to 10^5 copies) were prepared and analyzed in parallel. Distilled water (dw) was included as a negative control.

Figure 7 shows interactions of the p51 YMDD (SEQ ID NO: 8) motif of HIV-1 reverse transcriptase at the junction of the p51 palm, p51 connection and p51 fingers subdomains in the structure of the RT/DNA/dNTP complex (pdb code 1RTD). The Trp-
15 rich region is shown at the interface of the p51 and p66 subunits and proximal to the DNA-binding cleft.

Figure 8 shows infectivity for Trp motif mutants. (A): Lane 1: *trans*-Vprp51/p66 wild-type virus (15-20% of wild-type HIV-1). Normalized to 100%. Lane 2: Background control. Does not express p51 in the *vpr*-p51 reading-frame. Thus, it does not
20 incorporate p66 via Vpr-p51 and there is no active RT in the virion (other than minimal amounts of p66 that could get non-specifically incorporated). Lane 3-9: Mutants in the tryptophan-repeat motif (Trp-motif) of RT. This motif is found the connection subdomain of RT and is unique in having 6 Trp residues. These residues form a hydrophobic cluster of 12 tryptophans spanning the dimerization interface between the RT subunits (p51 and p66).
25 (B): Lane 1: *trans*-Vpr-p51/p66 wild-type virus. Lane 2: Background control. Does not express p51 in the *vpr*-p51 reading-frame. Thus, it does not incorporate p66 via Vpr-p51 and there is no active RT in the virion (other than minimal amounts of p66 that could get non-specifically incorporated). Lane 3-9: Mutants in the tryptophan-repeat motif (Trp-motif) of RT. The results of the RT assay are different in that clones like p51W401/p66 (lane 4)
30 have background levels of activity (Vpr-Dp51/p66) in this biochemical assay (Example 2) although this mutant is wild-type on infectivity analysis.

Figure 9 shows infectivity for p51W401-p66W410 dimer interface. (A) Lane 1: *trans*-Vpr-p51/p66 wild-type virus (15-20% of wild-type HIV-1). Normalized to 100%. Lane 2: Background control. Does not express p51 in the *vpr*-p51 reading-frame. Thus, it does not incorporate p66 via Vpr-p51 and there is no active RT in the virion (other than minimal amounts of p66 that could get non-specifically incorporated). The residues p51W401 and p66W410 are at the interface between p51 and p66 within interacting distance ($\sim 3\text{\AA}$) based on crystal structure. These residues were mutated both individually (lanes 3-6) and together (lanes 7-9). The single mutants do not have much effect on infectivity, while the double mutants have a greater effect. The p51/p66L234A (lane 10) and p51W401A/p66 (lane 11) are well-established dimerization defective mutants identified by biochemical and yeast-2-hybrid assay recognized in the field to be defective in RT assays (biochemical). It is quite clear from these controls that biochemical data do not accurately reflect what occurs in the virion. (B) Lane 1: *trans*-Vpr-p51/p66 wild-type virus (15-20% of wild-type HIV-1). Lane 2: Background control. Does not express p51 in the *vpr*-p51 reading-frame. Thus, it does not incorporate p66 via Vpr-p51 and there is no active RT in the virion (other than minimal amounts of p66 that could get non-specifically incorporated). The residues p51W401 and p66W410 are at the interface between p51 and p66 within interacting distance ($\sim 3\text{\AA}$) based on crystal structure. These residues were both mutated individually (lanes 3-6) and together (lanes 7-9). The single mutants to reduce RT (biochemical) activity to background levels (Vpr-Dp51/p66), while the double mutants have a greater effect and reduce the RT activity to negative control levels. The p51/p66L234A (lane 10) and p51W401A/p66 (lane 11), well-established dimerization defective mutants identified by biochemical and yeast-2-hybrid assay recognized in the field to be defective in RT assays (biochemical, Example 2) are also defective in our RT assay at negative control levels.

Figure 10 shows structural analysis of RT connection subdomain. (A) Alignment of Trp-motifs of primate lentiviruses. The Pol amino acid sequences of representative strains of primate lentiviruses were aligned using MegAlign (DNASTAR, Inc.). HIV-1 RT sequence (amino acids 395-415) is shown along with corresponding alignments for other indicated primate lentiviruses. (B) Ribbon representation of the p66 and p51 subunits in the crystal structure of the complex of HIV-1 RT with double-stranded DNA and incoming tenofovir-diphosphate (pdb file 1T05) (Tuske et al. *Nat. Struct. Mol. Biol.* 11:469-74 (2004)). For clarity, only the protein is shown. The tryptophan-rich motif and other p51

residues at the interface of the two subunits are shown in Van der Waals volumes. Residues W401 and W410 of the p66 subunit are shown at or near the interface also in Van der Waals volumes. Residue W401 of the p66 subunit and residue N363 of the p51 subunit are shown at interacting distance at the subunit interface. (C) Magnification of the area in the box shown in "B". Shown are the side-chains of residues of the tryptophan motif and of the interface that were mutated in this study. (D) Ribbon representation of the interface between p66 and p51. W410 of the p66 subunit is shown to have extensive interactions with residues of the p51 subunit (p51-N363, p51-W401, and p51-Y405).

Figure 11 shows the analysis of p51 Trp-motif mutants. M7 proviral DNA was transfected into 293T cells alone or together with wildtype or mutant *vpr-p51/p66* and *vpr-IN* expression plasmid DNAs. Transfection-derived virions were analyzed by immunoblotting for (A) RT (p51/p66) and (B) CA (p24). Expression of Vpr-p51 (C), p66 (D) and α -tubulin (E) in the transfected 293T was examined by immunoblotting. (F) Infectivity of p51 Trp-motif mutants. The infectivity of virions containing alanine substitutions in the p51 Trp-motif was analyzed using the TZM-bl reporter cell line as described in Example 4. Infectivity is expressed as a percentage of the wildtype *trans*-RT heterodimer (Vpr-p51/p66) complemented virions.

Figure 12 shows the analysis of Trp-motif residues located at the RT heterodimer interface. Trp-motif residues that lie within interacting distance at the dimer interface were mutated. The infectivity of virions containing single (A) or dual (B) mutations was analyzed by the TZM-bl reporter cell assay. Infectivity is expressed as a percentage of the wildtype *trans* heterodimer control.

Figure 13 shows the analysis of W401 and W410 mutations in proviral DNA. (A) The importance of RT Trp-motif residues W401 and W410 for viral infectivity was analyzed using the HIV-1 NL4-3 molecular clone. Infectivity was determined using TZM-bl reporter cells and the results are expressed as a percentage of wildtype NL4-3. Virions derived by transfection of the wildtype and mutant proviral DNAs were also analyzed by immunoblotting using mAbs to RT (B) and CA (C).

Figure 14 shows subunit specific analysis of the W401A mutant. The W401 residue was mutated in p51, p66 or p51 and p66. Transfection derived virions containing the respective mutant *trans* TR's, were analyzed for (A) infectivity on TZM-bl cells and (B) virion incorporation of p51 and p66 by immunoblotting. (C) Virion infectivity was

determined using the TZM-bl reporter cells (black bars) and the JLTRG-R5 reporter T cell line (white bars). Infectivity is expressed as a percentage of the wildtype *trans*-RT control.

Figure 15 shows the effect of NNRTIs on RT subunit interactions. Virions were generated by cotransfection of 293T cells with M7 and *trans*-RT dimerization-defective mutant plasmid *vpr-p51*^{W401A}/*p66*^{W401A}. The dimerization enhancing NNRTI EFV was added to the culture medium 12 h after DNA transfection at concentrations ranging from 0.01-1.0 μ M. The transfection-derived virions were collected 48 hours later and analyzed by immunoblot using mAbs to (A) RNase H and (B) CA.

Figure 16 shows an analysis of infectivity. Transfection-derived viruses were analyzed for infectivity using the TZM-bl reporter cell line as described in Example 1. Results are expressed as a percentage relative to an equal amount of wild-type SG3 virus.

Figure 17 shows an analysis of complementation using RT-deficient M7 virus. Increasing DNA concentrations of *vpr-p51/p66* or *vpr- Δ p51/p66* (ranging from 0.5 to 3.0 μ g) were transfected into 293T cells along with a constant amount of M7 (6 μ g) and *vpr-IN* (1 μ g). (A) Virion incorporation of *trans*-RT subunits. Transfection-derived virions were concentrated by ultracentrifugation, lysed and analyzed by immunoblotting using anti-RT MAb (8C4). (B) Analysis of infectivity. Virions were analyzed for infectivity using the TZM-bl reporter cell line. Results are expressed as a percentage of the wild-type SG3 virus.

Figure 18 shows alternative approaches for *trans*-heterodimeric RT complementation. (A) M7 virions derived by cotransfection with *vpr-p51/p66* and *vpr-IN* or *vpr-p51/p66-IN* were analyzed for infectivity by the TZM-bl assay. Results are expressed as percentage compared to the wild-type SG3 virus. (B and C) Immunoblot analysis. The virions were examined for (B) p66 and (C) CA using MAbs specific for either the RNase H subdomain (7E5) or CA, respectively. (D) Infectivity analysis of virions generated by expressing two monocistronic RT constructs, *vpr-p51* and *LTR-p66*. The + and - indicate the presence or absence of the plasmid included in the cotransfection, respectively. The amount of *vpr-p51* used was kept constant (3 μ g) while the *LTR-p66* was transfected at increasing concentrations (1, 2 and 3 μ g), indicated in parenthesis. M7 and *vpr-IN* were also included in the transfection. The transfection-derived virions were analyzed for infectivity using TZM-bl cells.

Figure 19 shows a distinction between Vpr-p51 and p66 by molecular mass. (A) Immunoblot analysis of virions derived by transfecting 293T cells with M7 and *vpr-p51/p66* expression plasmids containing the different sized Pro-coding sequences. The 8C4 MAb was used as a probe to detect both the p51 and p66 subunits. (B) The transfection-derived virions were examined for viral infectivity using the TZM-bl cells. Results are expressed as a percentage of the wild-type SG3 virus.

Figure 20 shows inhibition of trans-RT using NRTI and NNRTI. Virions derived by cotransfection with M7, *vpr-p51/p66* and *vpr-IN* were used to infect the TZM-bl reporter cell line. The two RT drugs, 3TC and nevirapine, were analyzed at concentrations of 0.04-1.0 μ M for 3TC and 1.0-25.0 μ M for nevirapine as described in Example 1. The results are expressed as a percentage of untreated virus.

Figure 21 shows enhancement of dimerization using Vpr-p51/p66. The RT heterodimerization enhancing drug, efavirenz (EFV), enhanced dimerization in a dose-dependent manner.

Figure 22 shows that the 2',5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl 3'-spiro-5''-(4''-amino-1'',2''-oxathiole 2'',2''-dioxide) (TSAO) exhibits inhibition characteristics similar to NNRTIs. The figure shows that TSAO can destabilize HIV-1 RT heterodimerization in p51 W401A/p66 W401A RT mutants.

Figure 23 shows that there exists an interaction at the dimer interface that is important for subunit interaction. An analysis of wild type, L234A, W398A, W401A and YMAA mutations was conducted in the context of the complete HIV-1 NL4-3 proviral clone. The wildtype or mutant proviral DNAs were transfected into 293T cells and progeny virions were analyzed for infectivity. The infectivity of virus containing a mutation was much less than that of wildtype, and was not rescued by efavirenz.

DETAILED DESCRIPTION OF THE INVENTION

The present invention may be understood more readily by reference to the following detailed description of preferred embodiments of the invention and the Examples included therein and to the Figures and their previous and following description.

DEFINITIONS

As used in the specification and the appended claims, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a small molecule” includes mixtures of one or more small molecules, and the like.

Ranges may be expressed herein as from “about” one particular value, and/or to “about” another particular value. When such a range is expressed, this includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint.

The terms “higher,” “increases,” “elevates,” “enhances” or “elevation” refer to increases above basal levels, or as compared to a control. The terms “low,” “lower,” “reduces,” “inhibits” “decreases” or “reduction” refer to decreases below basal levels, or as compared to a control. For example, basal levels are normal *in vivo* levels prior to, or in the absence of, the vector, or addition of an agent such as or another small molecule or ligand.

The term “test compound” is defined as any compound to be tested for its ability to interact with a selected cell.

The terms “control levels” or “control cells” are defined as the standard by which a change is measured, for example, the controls are not subjected to variables, but are instead subjected to a defined set of parameters in the absence of variables, or the controls are based on pre- or post-variable levels.

The terms “polypeptide,” “peptide,” and “protein” are used interchangeably throughout and are defined as sequences containing amino acids.

GENERAL

The biologically relevant, catalytically active form of human immunodeficiency virus type-1 (HIV-1) reverse transcriptase (RT) is a heterodimer consisting of a 51 kDa subunit and a 66 kDa subunit. Since p51 and p66 are derived from the same coding region, subunit-specific structure/function studies of RT have not been possible *in vivo*. RT has both

DNA polymerase and RNase H activities that are required to convert the single-stranded RNA viral genome into double-stranded DNA upon entry of the virus into host cells.

RT is translated and assembled into virions as part of a larger Gag-Pol polyprotein precursor (Pr¹⁶⁰Gag-Pol). Proteolytic processing of Pr160^{Gag-Pol} by the *pol*-encoded protease (PR) generates the mature heterodimeric form (p51/p66) of the RT enzyme (Freed and Martin, 2001; Telesnitsky and Goff, 1997). The N-terminal 440 amino acids of p51 and p66 are collinear. The p66 subunit contains the DNA polymerase and RNase H domains, while the p51 subunit lacks the RNase H domain (Hizi et al., 1988; Larder et al., 1987a; Prasad and Goff, 1989). Elucidation of the HIV-1 RT structure has shown that the polymerase domain of p51 and p66 can be further divided into the fingers, palm, thumb, and connection subdomains (Kohlstaedt et al., 1992). Although both p51 and p66 contain each of these subdomains, their relative arrangements differ markedly between the two subunits. Since these subunits are derived from the same coding region, a mutation in the polymerase coding region generates a heterodimer that contains a mutation in each subunit. However, as their structures are different in the heterodimer, the effect of these mutations on RT subunit structure/function is not equivalent (Arnold et al., 1992; Kohlstaedt et al., 1992). Thus, the heterodimeric nature of RT has previously had limited detailed molecular genetic analyses of the p51 and p66 subunit function.

Viral and foreign proteins can be incorporated into virions by exploiting viral accessory proteins, such as HIV/SIV proteins Vpr or Vpx, as targeting vehicles. By expressing the desired protein in *trans* as a fusion with Vpr or Vpx, its incorporation is brought about through an interaction between Vpr/Vpx and the p6 domain of the cognate Gag precursor polyprotein (Lu et al., 1993; Paxton et al., 1993; Wu et al., 1994). Using this approach, it has been shown that HIV-1 RT and IN functions can be provided when expressed in *trans* as Vpr fusion proteins, independently of Pr160^{Gag-Pol} (Liu et al., 1997; Wu et al., 1999; Wu et al., 1997).

Herein described is a *trans*-complementation approach that enables the function of the individual RT subunits to be analyzed in the context of an infectious virus. For example, by cotransfecting cells with RT-defective proviral DNA and an LTR-vpr-p51-IRES-p66 expression cassette, it was demonstrated that Vpr-p51 interacts with p66 and mediates virion incorporation of a Vpr-p51/p66 heterodimeric complex. The p51 subunit was expressed as a Vpr-p51 fusion protein that incorporates into HIV-1 virions through an

interaction between Vpr and the Gag precursor polyprotein. When coexpressed, p66 is specifically and selectively packaged as a Vpr-p51/p66 complex. Processing by the viral protease liberates Vpr and generates functional heterodimeric RT (p51/p66) that supports HIV-1 reverse transcription and virus infection (Example 1).

5 This approach was used to demonstrate that the YMDD aspartates of p66 are both required and sufficient for RT polymerase function, and that the p51 YMDD aspartates play a structural role that is required for viral cDNA synthesis in infected cells. By mutating D185 and D186 of either p51 or p66, the role of these residues, for the first time, in the context of an infectious virus, were studied. The results corroborate earlier findings that the
10 aspartates of p66 (YMDD) are required and sufficient for polymerase function of the RT heterodimer. Decreased viral DNA synthesis and infectivity was observed with certain p51 aspartate mutations (YMDD), indicating that both the occupancy and charge of these residues are important for RT function *in vivo*. These findings demonstrate detailed molecular genetic and biologic analyses of the RT subunits *in vivo*.

15 Furthermore, disclosed herein is a subunit-specific mutagenesis approach that enables precise molecular analysis of the heterodimer in the context of infectious HIV-1 particles (Example 4). The contributions of amino acids comprising the Trp-motif to RT subunit interaction and function were analyzed. The results revealed important inter- and intra-subunit interactions of residues in the Trp motif. A tryptophan cluster in p51 (W398,
20 W402, W406, W414), proximal to the interface, was found to be important for p51/p66 interaction and stability. At the dimer interface, residues W401, Y405 and N363 in p51 and W410 in p66 mediate inter-subunit interactions. The W401 residue is critical for RT dimerization (and therefore viral infectivity), exerting distinct effects in p51 and p66. The analysis of the RT heterodimerization enhancing non-nucleoside RT inhibitor (NNRTI),
25 efavirenz, indicates that the effects of drugs on RT dimer stability can be examined in human cells. Thus, subunit-specific molecular interactions that affect RT heterodimer function and virus infection *in vivo*, have been elucidated. Moreover, the ability to assess the effects of RT inhibitors on subunit interactions in a physiologically relevant context was demonstrated.

30 The first step in RT dimerization apparently involves interactions between hydrophobic residues in the connection subdomains of p51 and p66. This includes residues W401-W410 of p66 and residues P392-W401 of p51 (Rodriguez-Barrios et al. (2001);

Morris et al. *J. Biol. Chem.* 274:24941-6 (1999); Tachedjian et al. *J. Mol. Biol.* 326:381-96 (2003)). The connection subdomain is distinctive in having six tryptophans and a tyrosine between amino acids 398-414. This motif is well conserved among the primate lentiviruses, and has been appropriately dubbed the tryptophan-repeat motif (Trp-motif). In a yeast two-
5 hybrid approach to analyze Trp-motif mutations, residues p66^{W401} and p66^{W414} were shown to be involved in RT dimerization (Tachedjian et al., 2003). Mutagenesis of other aromatic amino acids that lie between these two residues did not affect subunit interaction. Since p66^{W401} and p66^{W414} are not located at the dimer interface, it appears that repositioning of structural elements between these residues accounted for their results.

10 Synthetic peptides corresponding to the connection subdomain (Trp-motif) have been reported to disrupt dimerization (Morris et al. (1999); Divita et al. (1994); Divita et al. *J. Biol. Chem.* 270:28642-6 (1995)). For example, a short peptide matching RT residues 395-404 was shown to inhibit heterodimerization *in vitro* and virus replication in cell culture (Morris et al. (1999)). Recent studies of nonnucleoside reverse transcriptase
15 inhibitors (NNRTI) have heightened interest in compounds that interfere with RT conformational flexibility as a novel drug design concept (Sarafianos et al. *Chem. Biol.* 6:R137-46 (1999); Hughes et al. *Proc. Natl. Acad. Sci. USA* 98:6991-2 (2001)). NNRTI are a group of small hydrophobic compounds with diverse structures that inhibit HIV-1 RT (see Balzarini et al. *Curr. Top. Med. Chem.* 4:921-44 (2004) for review). NNRTIs interact with
20 HIV-1 RT by binding to a site on the p66 subunit of the heterodimer. This results in both short-range and long-range distortions of the RT structure. NNRTIs have been shown to interfere directly with the global hinge-bending mechanism that controls the cooperative motions of the p66 fingers and thumb subdomains required for RT function (Temiz et al. *Proteins* 49:61-70 (2002); Madrid et al. *Proteins* 45:176-82 (2001)). In yeast, several
25 NNRTIs were shown to enhance p51/p66 subunit association as a result of a specific interaction of drug with p66 (Tachedjian et al. *Proc. Natl. Acad. Sci. USA* 98: 7188-93 (2001)).

The contribution of amino acid residues comprising the Trp-motif to RT subunit interaction and virus infection has been determined. Inter-subunit interactions between the
30 connection subdomains include W401, Y405 and N363 in p51 and W410 in p66, and mutation of these residues impairs RT function and virus infectivity. The W401 residue of the Trp-motif was found to be of central importance. Mutation of this amino acid simultaneously in both subunits is deleterious to RT dimerization and virus infection. The

RT heterodimerization enhancing drug, efavirenz (EFV), rescued this dimerization defect in a dose-dependent manner. Additionally, it was demonstrated that intra-subunit interactions between tryptophans comprising a hydrophobic cluster (W398, W402, W406, W414) proximal to the connection subdomain interface are important for p51/p66 subunit interaction and stability.

COMPOSITIONS

Disclosed are the components to be used to prepare the disclosed compositions as well as the compositions themselves to be used within the methods disclosed herein. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular plasmid is disclosed and discussed and a number of modifications that can be made to a number of molecules included in the plasmid are discussed, specifically contemplated is each and every combination and permutation of those molecules and the modifications that are possible unless specifically indicated to the contrary. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited each is individually and collectively contemplated meaning combinations, A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are considered disclosed. Likewise, any subset or combination of these is also disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E would be considered disclosed. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods.

Disclosed herein are plasmids comprising a fusion protein comprising a p51-containing DNA fragment fused in frame and a viral accessory protein, such as *vpr*. By expressing the desired protein in *trans* as a fusion with Vpr, for example, its incorporation is brought about through an interaction between Vpr and the p6 domain of the cognate Gag precursor polyprotein. The Vpr-p51 fusion includes the natural PR-RT cleavage site (PC), allowing processing by the viral protease and liberation of Vpr (Wu et al., 1997). Also

disclosed herein is an expression cassette comprising LTR-vpr-p51-IRES-p66, wherein the nucleic acid comprises SEQ ID NO: 1.

Also disclosed herein are vectors comprising a p66 subunit, a p51 subunit, and Vpr, wherein Vpr and p51 are expressed as a fusion protein. The p66 and p51 subunits can be expressed on the same, or on different, mRNAs.

Optionally, an internal ribosome entry site (IRES) can be placed downstream of *vpr-p51*, followed by the p66 coding sequence. IRES are *cis*-acting RNA sequences able to mediate internal entry of a sequence on some eukaryotic and viral messenger RNAs upstream of a translation initiation codon. Examples of useful IRES can be found at <http://ifr31w3.toulouse.inserm.fr/IRESdatabase>, herein incorporated by reference in its entirety for the disclosure of various IRES.

Transcription of *vpr-p51/p66* can then be placed under the control of a long terminal repeat (LTR), for example. LTRs are responsible for integration of the sequence into the host genome, initiation and enhancement of retroviral transcription, as well as transcriptional termination, and modulation of retroviral replication levels. Examples of LTRs useful with the plasmids described herein include SIV-LTR, HIV-1 LTR, and HIV-2 LTR, for example.

The plasmid can be incorporated into proviral clones that contain a deletion in RT. For example, the proviral clone pSG3^{FN} (Figure 1B) was used to study incorporation of the heterodimeric *trans*-RT into virions when coexpressed with the *vpr-p51/p66* expression plasmid (Example 1). The FN clone was selected for this purpose since it contains a deletion in RT that includes most of the RNase H region and extends 13 amino acids into the carboxyl-terminus of the p51 domain, however, any proviral clone can be used for this purpose. This created a defective RT, while the *pol* reading frame, including IN, remained open.

An expression plasmid including IN, such as *vpr-IN*, can also be included in conjunction with the plasmid disclosed herein. The M7 clone (pSG3^{FN}) does not express the IN protein, and integration of the nascent viral cDNA is required to detect infection. Moreover, IN is also required for efficient initiation of reverse transcription (Wu et al., 1999).

Effective *trans*-complementation requires expression of the two subunits (Vpr-p51 and p66), dimerization, and stable association of the p51 (Vpr-p51) and p66 subunits within the cytosol of the cell, specific interaction of Vpr with Pr55^{Gag}, incorporation of the Vpr-p51/p66 heterodimeric complex into virions, proteolytic cleavage to liberate Vpr from
5 p51/p66, and proper interaction of RT with the template-primer.

Also disclosed herein are cells comprising: (i) a vector comprising a p66 subunit, a p51 subunit, and Vpr, wherein Vpr and p51 are expressed as a fusion protein; (ii) and a reverse transcriptase deficient proviral DNA. One example of a cell that can be used is the 293T cell.

10 Also disclosed are cell lines stably transformed with the plasmid described herein. For example, the cell line can comprise an exogenous nucleic acid, the nucleic acid comprising vpr-p51/66. The cell line can express viral nucleic acids as well, and can be induced to express viral nucleic acids by contacting the cell with a stimulus. An example of such a stimulus includes, but is not limited to, tetracycline. Also disclosed are transgenic
15 animals expressing vpr-p51/66.

Homology/identity

It is understood that one way to define any known variants and derivatives or those that might arise, of the disclosed genes and proteins herein is through defining the variants and derivatives in terms of homology to specific known sequences. For example SEQ ID
20 NO: 1 sets forth a particular nucleic acid sequence encoding an expression protein and SEQ ID NO 2 sets forth a particular sequence of the protein encoded by *vpr*. Specifically disclosed are variants of these and other genes and proteins herein disclosed which have at least, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 percent homology to the stated sequence. Those of skill in the art
25 readily understand how to determine the homology of two proteins or nucleic acids, such as genes. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level. Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman Adv. Appl. Math. 2:
30 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48: 443 (1970), by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. U.S.A. 85: 2444 (1988), by computerized implementations of these algorithms

(GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. *Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are
5 herein incorporated by reference for at least material related to nucleic acid alignment.

Nucleic acids

There are a variety of molecules disclosed herein that are nucleic acid based, including for example the nucleic acids of the plasmid disclosed herein, as well as those that
10 encode the proteins disclosed herein, as well as various functional nucleic acids. The disclosed nucleic acids are made up of for example, nucleotides, nucleotide analogs, or nucleotide substitutes. Non-limiting examples of these and other molecules are discussed herein. It is understood that for example, when a vector is expressed in a cell, that the expressed mRNA will typically be made up of A, C, G, and U. Likewise, it is understood
15 that if, for example, an antisense molecule is introduced into a cell or cell environment through for example exogenous delivery, it is advantageous that the antisense molecule be made up of nucleotide analogs that reduce the degradation of the antisense molecule in the cellular environment.

Nucleotides and related molecules

20 A nucleotide is a molecule that contains a base moiety, a sugar moiety and a phosphate moiety. Nucleotides can be linked together through their phosphate moieties and sugar moieties creating an internucleoside linkage. The base moiety of a nucleotide can be adenin-9-yl (A), cytosin-1-yl (C), guanin-9-yl (G), uracil-1-yl (U), and thymin-1-yl (T). The sugar moiety of a nucleotide is a ribose or a deoxyribose. The phosphate moiety of a
25 nucleotide is pentavalent phosphate. An non-limiting example of a nucleotide would be 3'-AMP (3'-adenosine monophosphate) or 5'-GMP (5'-guanosine monophosphate).

A nucleotide analog is a nucleotide which contains some type of modification to either the base, sugar, or phosphate moieties. Modifications to nucleotides are well known in the art and would include for example, 5-methylcytosine (5-me-C), 5-hydroxymethyl
30 cytosine, xanthine, hypoxanthine, and 2-aminoadenine as well as modifications at the sugar or phosphate moieties.

Nucleotide substitutes are molecules having similar functional properties to nucleotides, but which do not contain a phosphate moiety, such as peptide nucleic acid (PNA). Nucleotide substitutes are molecules that will recognize nucleic acids in a Watson-Crick or Hoogsteen manner, but which are linked together through a moiety other than a phosphate moiety. Nucleotide substitutes are able to conform to a double helix type structure when interacting with the appropriate target nucleic acid.

It is also possible to link other types of molecules (conjugates) to nucleotides or nucleotide analogs to enhance for example, cellular uptake. Conjugates can be chemically linked to the nucleotide or nucleotide analogs. Such conjugates include but are not limited to lipid moieties such as a cholesterol moiety. (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989,86, 6553-6556),

A Watson-Crick interaction is at least one interaction with the Watson-Crick face of a nucleotide, nucleotide analog, or nucleotide substitute. The Watson-Crick face of a nucleotide, nucleotide analog, or nucleotide substitute includes the C2, N1, and C6 positions of a purine based nucleotide, nucleotide analog, or nucleotide substitute and the C2, N3, C4 positions of a pyrimidine based nucleotide, nucleotide analog, or nucleotide substitute.

A Hoogsteen interaction is the interaction that takes place on the Hoogsteen face of a nucleotide or nucleotide analog, which is exposed in the major groove of duplex DNA. The Hoogsteen face includes the N7 position and reactive groups (NH₂ or O) at the C6 position of purine nucleotides.

Sequences

There are a variety of sequences related to, for example, the plasmid described herein, as well as any other protein disclosed herein that are disclosed on Genbank, and these sequences and others are herein incorporated by reference in their entireties as well as for individual subsequences contained therein.

A variety of sequences are provided herein and these and others can be found in Genbank, at www.pubmed.gov. Those of skill in the art understand how to resolve sequence discrepancies and differences and to adjust the compositions and methods relating to a particular sequence to other related sequences.

Peptides

As discussed herein there are numerous variants of the vectors disclosed herein that are known and herein contemplated. In addition to the known functional variants there are derivatives of the proteins disclosed herein, such as Vpr, p51, or p66, which also function in the disclosed methods and compositions. Protein variants and derivatives are well understood to those of skill in the art and in can involve amino acid sequence modifications. For example, amino acid sequence modifications typically fall into one or more of three classes: substitutional, insertional or deletional variants. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Insertions ordinarily will be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of one to four residues. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. Typically, no more than about from 2 to 6 residues are deleted at any one site within the protein molecule. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the protein, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis.

Specifically, mutations can occur in p51, p66, Vpr, IRES, or any of the nucleic acids encoding these peptides. Mutations can also occur in the *env* gene of HIV, for example, which can optionally affect the infectivity of the virus. These mutations can be deletions, substitutions, or insertion mutations. The mutations can occur in RT and/or in IN. The mutations can also be point mutations.

Amino acid substitutions are typically of single residues, but can occur at a number of different locations at once; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. The mutations must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place. Such substitutions generally are made

in accordance with the following Tables 1 and 2 and are referred to as conservative substitutions.

TABLE 1: Amino Acid Abbreviations

Amino Acid	Abbreviations
alanine	AlaA
allosoleucine	Alle
arginine	ArgR
asparagine	AsnN
aspartic acid	AspD
cysteine	CysC
glutamic acid	GluE
glutamine	GlnK
glycine	GlyG
histidine	HisH
isoleucine	IleI
leucine	LeuL
lysine	LysK
phenylalanine	PheF
proline	ProP
pyroglutamic acid	Glu
Serine	SerS
Threonine	ThrT
Tyrosine	TyrY
Tryptophan	TrpW
Valine	ValV

TABLE 2: Amino Acid Substitutions
Original Residue Exemplary Conservative Substitutions, others are known in the art.
Ala-ser
Arg-lys, gln
Asn-gln; his
Asp-glu
Cys-ser
Gln-asn, lys
Glu-asp
Gly-pro
His-asn; gln
Ile-leu; val
Leu-ile; val
Lys-arg; gln;
Met-Leu; ile
Phe-met; leu; tyr
Ser-thr
Thr-ser
Trp-tyr
Tyr-trp; phe
Val-ile; leu

- Substantial changes in function or immunological identity are made by selecting
- 5 substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in

the protein properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by)
5 an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine, in this case, (e) by increasing the number of sites for sulfation and/or glycosylation.

For example, the replacement of one amino acid residue with another that is biologically and/or chemically similar is known to those skilled in the art as a conservative
10 substitution. For example, a conservative substitution would be replacing one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as, for example, Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such conservatively substituted variations of each explicitly disclosed sequence are included within the mosaic polypeptides provided herein.

15 Substitutional or deletional mutagenesis can be employed to insert sites for N-glycosylation (Asn-X-Thr/Ser) or O-glycosylation (Ser or Thr). Deletions of cysteine or other labile residues also may be desirable. Deletions or substitutions of potential proteolysis sites, e.g. Arg, is accomplished for example by deleting one of the basic residues or substituting one by glutaminyl or histidyl residues.

20 Certain post-translational derivatizations are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of
25 hydroxyl groups of seryl or threonyl residues, methylation of the o-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecular Properties*, W. H. Freeman & Co., San Francisco pp 79-86 [1983]), acetylation of the N-terminal amine and, in some instances, amidation of the C-terminal carboxyl.

It is understood that one way to define the variants and derivatives of the disclosed
30 proteins herein is through defining the variants and derivatives in terms of homology/identity to specific known sequences. For example, SEQ ID NO: 1 sets forth a particular nucleic acid sequence of a vector described herein, which encodes the Vpr and

p51 subunits; and SEQ ID NO: 2 sets forth a particular sequence of a Vpr protein.

Specifically disclosed are variants of these and other proteins herein disclosed which have at least, 70% or 75% or 80% or 85% or 90% or 95% homology to the stated sequence. Those of skill in the art readily understand how to determine the homology of two proteins. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. *Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment.

It is understood that the description of conservative mutations and homology can be combined together in any combination, such as embodiments that have at least 70% homology to a particular sequence wherein the variants are conservative mutations.

As this specification discusses various proteins and protein sequences it is understood that the nucleic acids that can encode those protein sequences are also disclosed. This would include all degenerate sequences related to a specific protein sequence, i.e. all nucleic acids having a sequence that encodes one particular protein sequence as well as all nucleic acids, including degenerate nucleic acids, encoding the disclosed variants and derivatives of the protein sequences. Thus, while each particular nucleic acid sequence may not be written out herein, it is understood that each and every sequence is in fact disclosed and described herein through the disclosed protein sequence.

It is understood that there are numerous amino acid and peptide analogs which can be incorporated into the disclosed compositions. For example, there are numerous D amino

acids or amino acids which have a different functional substituent than the amino acids shown in Table 1 and Table 2. The opposite stereo isomers of naturally occurring peptides are disclosed, as well as the stereo isomers of peptide analogs. These amino acids can readily be incorporated into polypeptide chains by charging tRNA molecules with the amino acid of choice and engineering genetic constructs that utilize, for example, amber codons, to insert the analog amino acid into a peptide chain in a site specific way (Thorson et al., Methods in Molec. Biol. 77:43-73 (1991), Zoller, Current Opinion in Biotechnology, 3:348-354 (1992); Ibba, Biotechnology & Genetic Engineering Reviews 13:197-216 (1995), Cahill et al., TIBS, 14(10):400-403 (1989); Benner, TIB Tech, 12:158-163 (1994); Ibba and Hennecke, Bio/technology, 12:678-682 (1994) all of which are herein incorporated by reference at least for material related to amino acid analogs).

Molecules can be produced that resemble peptides, but which are not connected via a natural peptide linkage. For example, linkages for amino acids or amino acid analogs can include $\text{CH}_2\text{NH--}$, $\text{--CH}_2\text{S--}$, $\text{--CH}_2\text{--CH}_2\text{--}$, --CH=CH-- (cis and trans), $\text{--COCH}_2\text{--}$, $\text{CH(OH)CH}_2\text{--}$, and $\text{--CHH}_2\text{SO--}$ (These and others can be found in Spatola, A. F. in Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3, Peptide Backbone Modifications (general review); Morley, Trends Pharm Sci (1980) pp. 463-468; Hudson, D. et al., Int J Pept Prot Res 14:177-185 (1979) ($\text{--CH}_2\text{NH--}$, $\text{CH}_2\text{CH}_2\text{--}$); Spatola et al. Life Sci 38:1243-1249 (1986) ($\text{--CH H}_2\text{--S--}$); Hann J. Chem. Soc Perkin Trans. I 307-314 (1982) (--CH--CH-- , cis and trans); Almquist et al. J. Med. Chem. 23:1392-1398 (1980) ($\text{--COCH}_2\text{--}$); Jennings-White et al. Tetrahedron Lett 23:2533 (1982) ($\text{--COCH}_2\text{--}$); Szelke et al. European Appln, EP 45665 CA (1982): 97:39405 (1982) ($\text{CH(OH)CH}_2\text{--}$); Holladay et al. Tetrahedron. Lett 24:4401-4404 (1983) ($\text{--C(OH)CH}_2\text{--}$); and Hruby Life Sci 31:189-199 (1982) ($\text{--CH}_2\text{--S--}$); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is $\text{--CH}_2\text{NH--}$. It is understood that peptide analogs can have more than one atom between the bond atoms, such as β -alanine, γ -aminobutyric acid, and the like.

Amino acid analogs and analogs and peptide analogs often have enhanced or desirable properties, such as, more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others.

D-amino acids can be used to generate more stable peptides, because D amino acids are not recognized by peptidases and such. Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) can be used to generate more stable peptides. Cysteine residues can be used to cyclize or attach two or more peptides together. This can be beneficial to constrain peptides into particular conformations. (Rizo and Gierasch Ann. Rev. Biochem. 61:387 (1992), incorporated herein by reference).

Functional Nucleic Acids

Functional nucleic acids are nucleic acid molecules that have a specific function, such as binding a target molecule or catalyzing a specific reaction. The compositions and methods described herein can be used with any functional nucleic acid. Functional nucleic acid molecules can be divided into the following categories, which are not meant to be limiting. For example, functional nucleic acids include antisense molecules, aptamers, ribozymes, triplex forming molecules, and external guide sequences. The functional nucleic acid molecules can act as effectors, inhibitors, modulators, and stimulators of a specific activity possessed by a target molecule, or the functional nucleic acid molecules can possess a de novo activity independent of any other molecules.

Functional nucleic acid molecules can interact with any macromolecule, such as DNA, RNA, polypeptides, or carbohydrate chains. Thus, functional nucleic acids can interact with the mRNA of HIV or the genomic DNA of the subject, or they can interact with the polypeptide of the compositions disclosed herein. Often functional nucleic acids are designed to interact with other nucleic acids based on sequence homology between the target molecule and the functional nucleic acid molecule. In other situations, the specific recognition between the functional nucleic acid molecule and the target molecule is not based on sequence homology between the functional nucleic acid molecule and the target molecule, but rather is based on the formation of tertiary structure that allows specific recognition to take place.

Antisense molecules are designed to interact with a target nucleic acid molecule through either canonical or non-canonical base pairing. The interaction of the antisense molecule and the target molecule is designed to promote the destruction of the target molecule through, for example, RNaseH mediated RNA-DNA hybrid degradation. Alternatively the antisense molecule is designed to interrupt a processing function that

normally would take place on the target molecule, such as transcription or replication.

Antisense molecules can be designed based on the sequence of the target molecule.

Numerous methods for optimization of antisense efficiency by finding the most accessible regions of the target molecule exist. Exemplary methods would be in vitro selection

5 experiments and DNA modification studies using DMS and DEPC. It is preferred that antisense molecules bind the target molecule with a dissociation constant (k_d) less than or equal to 10^{-6} , 10^{-8} , 10^{-10} , or 10^{-12} . A representative sample of methods and techniques which aid in the design and use of antisense molecules can be found in the following non-limiting list of United States patents: 5,135,917, 5,294,533, 5,627,158, 5,641,754, 5,691,317,
10 5,780,607, 5,786,138, 5,849,903, 5,856,103, 5,919,772, 5,955,590, 5,990,088, 5,994,320, 5,998,602, 6,005,095, 6,007,995, 6,013,522, 6,017,898, 6,018,042, 6,025,198, 6,033,910, 6,040,296, 6,046,004, 6,046,319, and 6,057,437.

Aptamers are molecules that interact with a target molecule, preferably in a specific way. Typically aptamers are small nucleic acids ranging from 15-50 bases in length that
15 fold into defined secondary and tertiary structures, such as stem-loops or G-quartets. Aptamers can bind small molecules, such as ATP (United States patent 5,631,146) and theophiline (United States patent 5,580,737), as well as large molecules, such as reverse transcriptase (United States patent 5,786,462) and thrombin (United States patent 5,543,293). Aptamers can bind very tightly with k_d s from the target molecule of less than
20 10^{-12} M. It is preferred that the aptamers bind the target molecule with a k_d less than 10^{-6} , 10^{-8} , 10^{-10} , or 10^{-12} . Aptamers can bind the target molecule with a very high degree of specificity. For example, aptamers have been isolated that have greater than a 10000 fold difference in binding affinities between the target molecule and another molecule that differ at only a single position on the molecule (United States patent 5,543,293). It is preferred
25 that the aptamer have a k_d with the target molecule at least 10, 100, 1000, 10,000, or 100,000 fold lower than the k_d with a background binding molecule. It is preferred when doing the comparison for a polypeptide for example, that the background molecule be a different polypeptide. Representative examples of how to make and use aptamers to bind a variety of different target molecules can be found in the following non-limiting list of
30 United States patents: 5,476,766, 5,503,978, 5,631,146, 5,731,424, 5,780,228, 5,792,613, 5,795,721, 5,846,713, 5,858,660, 5,861,254, 5,864,026, 5,869,641, 5,958,691, 6,001,988, 6,011,020, 6,013,443, 6,020,130, 6,028,186, 6,030,776, and 6,051,698.

Ribozymes are nucleic acid molecules that are capable of catalyzing a chemical reaction, either intramolecularly or intermolecularly. Ribozymes are thus catalytic nucleic acid. It is preferred that the ribozymes catalyze intermolecular reactions. There are a number of different types of ribozymes that catalyze nuclease or nucleic acid polymerase type reactions which are based on ribozymes found in natural systems, such as hammerhead ribozymes, (for example, but not limited to the following United States patents: 5,334,711, 5,436,330, 5,616,466, 5,633,133, 5,646,020, 5,652,094, 5,712,384, 5,770,715, 5,856,463, 5,861,288, 5,891,683, 5,891,684, 5,985,621, 5,989,908, 5,998,193, 5,998,203, WO 9858058 by Ludwig and Sproat, WO 9858057 by Ludwig and Sproat, and WO 9718312 by Ludwig and Sproat) hairpin ribozymes (for example, but not limited to the following United States patents: 5,631,115, 5,646,031, 5,683,902, 5,712,384, 5,856,188, 5,866,701, 5,869,339, and 6,022,962), and tetrahymena ribozymes (for example, but not limited to the following United States patents: 5,595,873 and 5,652,107). There are also a number of ribozymes that are not found in natural systems, but which have been engineered to catalyze specific reactions de novo (for example, but not limited to the following United States patents: 5,580,967, 5,688,670, 5,807,718, and 5,910,408). Preferred ribozymes cleave RNA or DNA substrates, and more preferably cleave RNA substrates. Ribozymes typically cleave nucleic acid substrates through recognition and binding of the target substrate with subsequent cleavage. This recognition is often based mostly on canonical or non-canonical base pair interactions. This property makes ribozymes particularly good candidates for target specific cleavage of nucleic acids because recognition of the target substrate is based on the target substrates sequence. Representative examples of how to make and use ribozymes to catalyze a variety of different reactions can be found in the following non-limiting list of United States patents: 5,646,042, 5,693,535, 5,731,295, 5,811,300, 5,837,855, 5,869,253, 5,877,021, 5,877,022, 5,972,699, 5,972,704, 5,989,906, and 6,017,756.

Triplex forming functional nucleic acid molecules are molecules that can interact with either double-stranded or single-stranded nucleic acid. When triplex molecules interact with a target region, a structure called a triplex is formed, in which there are three strands of DNA forming a complex dependant on both Watson-Crick and Hoogsteen base-pairing. Triplex molecules are preferred because they can bind target regions with high affinity and specificity. It is preferred that the triplex forming molecules bind the target molecule with a k_d less than 10^{-6} , 10^{-8} , 10^{-10} , or 10^{-12} . Representative examples of how to make and use triplex forming molecules to bind a variety of different target molecules can be found in the

following non-limiting list of United States patents: 5,176,996, 5,645,985, 5,650,316, 5,683,874, 5,693,773, 5,834,185, 5,869,246, 5,874,566, and 5,962,426.

External guide sequences (EGSs) are molecules that bind a target nucleic acid molecule forming a complex, and this complex is recognized by RNase P, which cleaves the target molecule. EGSs can be designed to specifically target a RNA molecule of choice. RNase P aids in processing transfer RNA (tRNA) within a cell. Bacterial RNase P can be recruited to cleave virtually any RNA sequence by using an EGS that causes the target RNA:EGS complex to mimic the natural tRNA substrate. (WO 92/03566 by Yale, and Forster and Altman, Science 238:407-409 (1990)).

Similarly, eukaryotic EGS/RNase P-directed cleavage of RNA can be utilized to cleave desired targets within eukaryotic cells. (Yuan et al., Proc. Natl. Acad. Sci. USA 89:8006-8010 (1992); WO 93/22434 by Yale; WO 95/24489 by Yale; Yuan and Altman, EMBO J 14:159-168 (1995), and Carrara et al., Proc. Natl. Acad. Sci. (USA) 92:2627-2631 (1995)). Representative examples of how to make and use EGS molecules to facilitate cleavage of a variety of different target molecules be found in the following non-limiting list of United States patents: 5,168,053, 5,624,824, 5,683,873, 5,728,521, 5,869,248, and 5,877,162.

METHODS

Also disclosed herein are methods of screening for a compound that inhibits viral reverse transcriptase comprising: a) contacting a cell comprising (i) a plasmid which expresses a fusion protein comprising a p66 subunit, a p51 subunit, and Vpr, (ii) and a reverse transcriptase deficient proviral DNA with the compound, and b) comparing the level of viral infectivity in the presence of the compound with the level of viral infectivity in the absence of the compound, wherein a decreased level of infectivity in the presence of the compound indicates that the compound inhibits reverse transcriptase.

The compositions and methods described herein can be used to treat retroviruses, and in particular lentiviruses. Lentiviruses share several molecular and pathogenic features that set them apart from other retroviruses. These include virus encoded regulatory proteins to stimulate viral gene expression, synthesis of multiply spliced mRNAs and chronic infection associated with slow development of disease. Lentiviruses include, but are not limited to, HIV-1, HIV-2 and SIV. In the methods described therein, the HIV or SIV

particles can be derived by genes expressed in the cell, wherein the genes contain one or more nucleotide mutations. Examples of these specific mutations can be found in Example 1.

5 The p51 and p66 subunits can be expressed on the same or on different messenger RNAs. Furthermore, expression of Vpr-p51 can incorporate the p66 protein into viral particles. The plasmid can also express an internal ribosome entry site (IRES), as described above.

Also disclosed are methods of screening for a compound that inhibits dimerization of a p66 subunit polypeptide of reverse transcriptase and a p51 subunit polypeptide of reverse transcriptase comprising: a) contacting a cell comprising (i) a plasmid which expresses a fusion protein comprising a p66 subunit, a p51 subunit, and Vpr, (ii) and a reverse transcriptase deficient proviral DNA with the compound, and b) comparing the level of complex formation in the presence of the compound with the level of complex formation in the absence of the compound, a lower level of complex formation indicating that the compound inhibits dimerization of the p66 subunit and a p51 subunit.

Also disclosed are methods of screening for a compound that enhances dimerization of a p66 subunit polypeptide of reverse transcriptase and a p51 subunit polypeptide of reverse transcriptase comprising: a) contacting a cell comprising (i) a plasmid which expresses a fusion protein comprising a p66 subunit, a p51 subunit, and Vpr, (ii) and a reverse transcriptase deficient proviral DNA with the compound, and b) comparing the level of complex formation in the presence of the compound with the level of complex formation in the absence of the compound, a lower level of complex formation indicating that the compound enhances dimerization of the p66 subunit and a p51 subunit. Examples of compounds that inhibit reverse transcriptase by enhancing subunit dimerization include, but are not limited to, NNRTI.

Also disclosed is a method of inhibiting viral reverse transcriptase comprising contacting (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of the compound identified by the method described above, thereby inhibiting viral reverse transcriptase.

Also disclosed is a method of inhibiting dimerization of a p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase, which comprises contacting either (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of the compound identified by the method described above, thereby inhibiting dimerization of the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase.

Also disclosed is a method of enhancing dimerization of a p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase, which comprises contacting either (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of the compound identified by the method described above, thereby enhancing dimerization of the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase.

In the methods described above, HIV-1 reverse transcriptase can be present in a subject, a eukaryotic cell, or a prokaryotic cell, for example.

The compounds disclosed herein can be NNRTIs. NNRTIs are a chemically diverse group of largely hydrophobic compounds that inhibit HIV-1 RT by binding in a hydrophobic pocket near the polymerase active site in the p66 subunit. NNRTIs have been described that can either stabilize or destabilize the RT heterodimer. Various NNRTIs have also been found to induce increased β -gal activity in the yeast two-hybrid system, due to enhanced RT subunit association 30. In particular, efavirenz binding to the NNRTI hydrophobic pocket enhanced RT heterodimerization, including RT with p51/p66 W401 mutations.

Additionally, both the 2',5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl 3'-spiro-5''-(4''-amino-1'',2''-oxathiole 2'',2''-dioxide) (TSAO) thymine derivatives and the *N*-acylhydrazones are classes of compounds that show inhibition characteristics similar to NNRTIs. Although these drugs may not bind to the well-defined NNRTI binding pocket of HIV-1 RT, they bind to a region of RT close to and partially overlapping this site.

Furthermore, in the presence of a denaturant like urea these compounds have been shown to destabilize HIV-1 RT heterodimerization. Results show a dose-dependent enhancement of dimerization of the p51^{W401A}/p66^{W401A} RT mutant in the presence of efavirenz. (Example 4).

As used throughout, by a "subject" is meant an individual. Thus, the "subject" can include domesticated animals, such as cats, dogs, etc., livestock (e.g., cattle, horses, pigs, sheep, goats, etc.), laboratory animals (e.g., mouse, rabbit, rat, guinea pig, etc.) and birds. Preferably, the subject is a mammal such as a primate, and, more preferably, a human.

5 The methods of screening described herein are useful with high throughput screening methods. Screening optionally takes place in multi-well plates. Multi-well plates are standard in the art and come in a variety of sizes and shapes. For example, the multi-well plate can be 24, 48, or 96 well plates. Such screening assays can be automated or further modified for high throughput analysis. For high throughput screening, each well can include numerous test
10 components. If a positive reaction is detected in a well, the screening is repeated with one of the test compounds contained in a single well.

Optionally, reverse transcriptase containing (vpr-p51/p66) virus particles can be made that either lack Env or contain either autologous or heterologous Env derived by pseudotyping. Wei shows this with autologous Env, (Wei, X., J. M. Decker, H. Liu, Z.
15 Zhang, R. B. Arani, J. M. Kilby, M. S. Saag, X. Wu, G. M. Shaw, and J. C. Kappes. 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob Agents Chemother 46:1896-905; Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M.
20 Shaw. 2003. Antibody neutralization and escape by HIV-1. Nature 422:307-12; both herein incorporated in their entireties for the teaching of autologous Env); while Wu shows this with the VSV-G Env (Wu, X., J. K. Wakefield, H. Liu, H. Xiao, R. Kralovics, J. T. Prchal, and J. C. Kappes. 2000. Development of a novel trans-lentiviral vector that affords predictable safety. Mol Ther 2:47-55, herein incorporated by reference in its entirety for its
25 teaching of VSV-G Env). The Wei citations describe how env minus or env mutant virus can be rendered infectious by providing an envelope glycoprotein in *trans*.

COMPOUNDS AND METHODS OF MAKING

Also disclosed herein are methods of making a pharmaceutical composition which comprises: a) determining whether a compound inhibits reverse transcriptase by the
30 methods described herein; and b) admixing the compound with a pharmaceutically acceptable carrier.

Also disclosed are compounds identified by the methods described herein, as well as compositions comprising the compounds identified by the methods described herein. Such compositions can also comprise a carrier. The compound can be capable of inhibiting HIV-1. Optionally, the compound can be a nonnucleoside reverse transcriptase inhibitor, or a
5 nucleoside reverse transcriptase inhibitor. These compounds are known to those of ordinary skill in the art.

The compositions of the invention can be administered *in vivo* in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable. Thus, the material may be administered to a subject,
10 without causing undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art.

15 The compositions identified by the methods disclosed herein can be administered orally, parenterally (e.g., intravenously), by intramuscular injection, intravenously, subcutaneously, intramuscularly, by intraperitoneal injection, transdermally, extracorporeally, topically or the like, including topical intranasal administration or administration by inhalant. topically or by liposome-mediated delivery. As used herein,
20 "topical intranasal administration" means delivery of the compositions into the nose and nasal passages through one or both of the nares and can comprise delivery by a spraying mechanism or droplet mechanism, or through aerosolization of the small molecule or ligand. Administration of the compositions by inhalant can be through the nose or mouth via delivery by a spraying or droplet mechanism. Delivery can also be directly to any area of
25 the respiratory system (e.g., lungs) via intubation.

The dosage of the compositions required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the infection being treated, the particular active agent used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for every composition.

30 However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein.

The materials may be in solution, suspension (for example, incorporated into microparticles, liposomes, or cells). These may be targeted to a particular cell type via antibodies, receptors, or receptor ligands.

Suitable carriers and their formulations are described in *Remington: The Science and Practice of Pharmacy* (19th ed.) (ed. A.R. Gennaro, Mack Publishing Company, Easton, PA 1995.) Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically-acceptable carrier include, but are not limited to, saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8.5, and more preferably from about 7.8 to about 8.2. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of composition being administered.

Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration of drugs to humans, including solutions such as sterile water, saline, and buffered solutions at physiological pH. Other compounds will be administered according to standard procedures used by those skilled in the art.

Pharmaceutical compositions may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the molecule of choice. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, anti-inflammatory agents, anesthetics, and the like.

The terms "effective amount" and "effective dosage" are used interchangeably. The term "effective amount" is defined as any amount necessary to produce a desired physiologic response. Effective amounts and schedules for administering the compositions may be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for the administration of the compositions are those large enough to produce the desired effect in which the symptoms or disorder are affected. The dosage should not be so large as to cause substantial adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex, type of disease and extent of the disease in the patient, route of

administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any contraindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature
5 for appropriate dosages for given classes of pharmaceutical products.

Parenteral administration of a nucleic acid or vector to a subject is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral
10 administration involves use of a slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein. For additional discussion of suitable formulations and various routes of administration of therapeutic compounds, see, e.g., *Remington: The Science and Practice of Pharmacy* (19th ed.) ed. A.R. Gennaro, Mack Publishing Company, Easton, PA 1995.

15 Also, provided are kits for screening for compounds comprising a plasmid which expresses a fusion protein comprising a p66 subunit, a p51 subunit, and Vpr, and a reverse transcriptase deficient proviral DNA. Also provided are kits comprising a cell comprising the plasmid. Also provided are kits for treating viral infections comprising a composition identified by the methods disclosed herein.

20 The present invention is more particularly described in the following examples, which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as
25 limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to
30 be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect

to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

EXAMPLES

5 **Example 1: Subunit-Specific Analysis of the Human Immunodeficiency Virus Type-1 Reverse Transcriptase *In Vivo***

Expression and virion incorporation of heterodimeric RT in trans

For independent expression of the p51 and p66 subunits, the bicistronic pLR2P-vpr-p51-IRES-p66 (*vpr-p51/p66*) expression plasmid (abbreviations can be found in Table III) was constructed (Figure 1A). The p51-containing DNA fragment was fused in frame with that of *vpr*. The Vpr-p51 fusion included the natural PR-RT cleavage site (PC), allowing processing by the viral protease and liberation of Vpr (Wu et al., 1997). The encephalomyocarditis virus internal ribosome entry site (IRES) was placed downstream of *vpr-p51*, followed by the p66 coding sequence. Transcription of *vpr-p51/p66* was under control of the HIV-2 LTR (Wu et al., 1995).

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TABLE III. Abbreviations for plasmids used in study

Plasmid	Abbreviation
pSG3 ^{wt}	SG3
pSG3 ^{S-RT}	S-RT
pSG3 ^{FN}	FN
pSG3 ^{M7}	M7
pLR2P-vpr-p66	<i>vpr-p66</i>
pLR2P-vpr-Δp51-IRES-p66	<i>vpr-Δp51/p66</i>
pLR2P-vpr-p51-IRES-p66	<i>vpr-p51/p66</i>
pLR2P-vpr-p51-IRES- p66 ^{YMNN}	<i>vpr-p51/p66^{NN}</i>
pLR2P-vpr-p51 ^{YMNN} -IRES- p66	<i>vpr-p51^{NN}/p66</i>
pLR2P-vpr-p51 ^{YMAA} -IRES- p66	<i>vpr-p51^{AA}/p66</i>
pLR2P-vpr-p51 ^{YMEE} -IRES- p66	<i>vpr-p51^{EE}/p66</i>
pLR2P-vpr-p51 ^{YMKK} -IRES- p66	<i>vpr-p51^{KK}/p66</i>
pLR2P-vpr-IN	<i>vpr-IN</i>

The proviral clone pSG3^{FN} (FN) (Figure 1B) was used to study incorporation of the heterodimeric *trans*-RT into virions when coexpressed with the *vpr-p51/p66* expression
5 plasmid. The FN clone was selected for this purpose since it contains a deletion in RT that includes most of the RNase H region and extends 13 amino acids into the carboxyl-terminus of the p51 domain. This created a defective RT, while the *pol* reading frame, including IN, remained open. This overall strategy for studying subunit-specific RT function in the context of infectious virus is illustrated in Figure 2. Effective *trans*-complementation would
10 require expression of the two subunits (Vpr-p51 and p66), dimerization and stable

association of the p51 (Vpr-p51) and p66 subunits within the cytosol of the cell, specific interaction of Vpr with Pr55^{Gag}, incorporation of the Vpr-p51/p66 heterodimeric complex into virions, proteolytic cleavage to liberate Vpr from p51/p66, and proper interaction of RT with the template-primer.

5 It was first determined whether the Vpr-p51 fusion protein could selectively incorporate p66 into virions. Virions derived by cotransfecting 293T cells with *vpr-p51/p66* and FN were analyzed by immunoblot analysis. Using polyclonal anti-RT antiserum, two predominant proteins detected were consistent with the molecular masses of p51 and p66 (Figure 3A, lane 6), and comigrated with those detected using SG3 virions (lane 1). Neither
10 protein was detected using the RT-minus pSG3^{S-RT} (S-RT) virus (lane 2). Detection of the 51 kDa polypeptide with polyclonal anti-RT antibody showed that Vpr-p51 was packaged and processing by the viral protease liberated p51. The detection of the 66 kDa polypeptide showed incorporation of p66, however, the molecular mass of the unprocessed Vpr-p51 fusion protein is similar to that of p66. Therefore, a monoclonal antibody specific to the
15 RNase H domain of p66 was used as a probe and confirmed incorporation of the *trans*-p66 subunit into virions (Figure 3B, lane 6). As controls, virions produced by transfecting 293T cells with FN alone and FN in combination with the pLR2P-vprRT (*vpr-p66*) expression plasmid were analyzed. A protein comigrating with p51 that likely represents the truncated RT protein product (p51Δ13) was detected in virions generated by FN (lane 3). When the
20 *vpr-p66* expression plasmid was cotransfected with FN, p66, p51 and unprocessed Vpr-p66 were detected in virions (lane 4). To determine whether the incorporation of p66 was mediated selectively by the Vpr-p51 fusion protein, virus derived by cotransfecting 293T cells with FN and pLR2P-vpr-Δp51-IRES-p66 (*vpr-Δp51/p66*) was analyzed (lane 5). The
25 *vpr-Δp51/p66* expression plasmid abrogates expression of the p51-coding region without affecting p66 expression. Using both polyclonal and monoclonal antibodies, a protein with a molecular mass equal to that of p66 was detected, showing that p66 incorporation, at least in part, was not selectively mediated by Vpr-p51 (lane 5). Immunoblot analysis using a monoclonal antibody against CA confirmed that approximately the same amount of each virus was analyzed (Figure 3C).

30 To examine whether the *trans*-heterodimeric RT could rescue the defect in FN infectivity, the transfection-derived virions were analyzed using the single cycle TZM-bl reporter assay. In three independent experiments, cotransfection of the *vpr-p51/p66*

expression plasmid rescued FN infectivity to levels of 15-20% compared to wildtype SG3 virus (Fig. 16, lane 6). Virus derived by cotransfecting 293T cells with FN and vpr-p66 exhibited a similar level of infectivity (lane 4). The infectivity of FN virion derived by cotransfection with *vpr-Δp51/p66* was approximately 3.5% of wildtype SG3 (lane 5). The RT-defective M7 and FN viruses had no detectable infectivity (lanes 2 and 3, respectively). These results showed that the heterodimeric trans-RT was to catalyze HIV-1 reverse transcription.

Specific packaging of heterodimeric RT

The strategy used for analyzing RT subunit function necessitates Vpr-p51-mediated selective incorporation of p66. Non-specifically packaged p66 can form p66/p66 homodimers and through proteolytic processing generate p51/p66 RT heterodimers, thus confounding the analysis of subunit-specific mutations. One explanation for the non-specific packaging of p66 observed in Figure 3 was translational read-through of the TAA stop codon placed at the 5' end of p51 in the *vpr-Δp51/p66* expression plasmid. A second explanation was that the *trans*-p66 protein may associate intracellularly with the Gag-Pol polyprotein encoded by FN. Therefore, the pSG3^{M7} (M7) proviral clone was constructed. M7 has multiple mutations in the RT and IN coding regions (Figure 4A) and was constructed to minimize the chance of encoding functional RT and IN, including that which conceivably could be generated via intermolecular genetic recombination with the *vpr-p51/p66* plasmid. Virus generated by cotransfection of M7 with *vpr-p51/p66* contained the p51 and p66 proteins, detectable with monoclonal anti-RT antibody (Figure 4B, lane 5). In contrast to virus generated by FN (Figure 3), virus generated by cotransfecting 293T cells with M7 and *vpr-Δp51/p66* did not contain detectable p66 (lane 4). Probing blots with p66 monoclonal antibody confirmed selective, Vpr-p51-mediated packaging of p66 (Figure 4C, lane 5). By probing a replica blot with monoclonal antibody against CA, it was confirmed that approximately the same amount of each virus was analyzed, and that the M7 virus did not have detectable abnormalities in either virion assembly or maturation (Figure 4D). These results demonstrated that the Vpr-p51 fusion protein can selectively incorporate the p66 RT subunit into HIV-1 virions. Moreover, they indicate that the p51/p66 heterodimer is relatively stable, subsequent to virion incorporation. If not, free p66 might be expected to form homodimers that would be processed by viral PR, resulting in excess p51. However, Figure 4B shows that a similar amount of each subunit was present in the M7 virions.

Heterodimeric trans-RT rescues the infectivity of RT-deficient virus

To determine if the heterodimeric *trans*-RT was functional, the M7 proviral construct was cotransfected into 293T cells with *vpr-p51/p66* and *vpr-IN*. The *vpr-IN* expression plasmid was included since the M7 clone does not express the IN protein and integration of the nascent viral cDNA is required to detect infection using the TZM-bl reporter cell line. Moreover, IN is also required for efficient initiation of reverse transcription (Wu et al., 1999). In three independent experiments, virus infectivity was rescued to about 15% of that of wild-type virus (Figure 5, lane 5). Virus derived by cotransfecting 293T cells with M7, *vpr-p66* and *vpr-IN* exhibited a similar level of infectivity (lane 3), consistent with earlier reports (Wu et al., 1997). The infectivity of M7 virus derived by cotransfection with *vpr-Δp51/p66* and *vpr-IN* was less than 0.05% of wild-type virus (lane 4), or 0.2% compared with virus complemented with *vpr-p51/p66*. These results demonstrated that the heterodimeric *trans*-RT is functional, and with the M7 proviral clone, minimal complementation of virus infectivity was due to non-Vpr-p51 mediated packaging of p66. Furthermore, virus infectivity was not efficiently complemented without the IN protein (lane 6).

Subunit-specific analysis of the YMDD motif

There exists a preponderance of evidence from biochemical and structural studies that shows HIV-1 reverse transcription is catalyzed by the p66 subunit of RT. However, the function of D185 and D186 in the p51 and p66 subunits, respectively, has not been directly tested in the context of an infectious virus. To study the function of these aspartate residues in one subunit independently of the other, either the p66 or the p51 coding region of the *vpr-p51/p66* plasmid was mutated in both aspartates of the YMDD motif. Virus was analyzed for infectivity using the TZM-bl reporter cell line and for DNA synthesis following acute infection of JC53 cells. Virus containing the p51/p66^{YMNN} mutant RT with Asp185Asn and Asp186Asn mutations in p66 was severely defective in infectivity (Figure 6A, lane 3). Analysis of infected cells for viral DNA revealed a severe defect in reverse transcription (Figure 6B and C, lanes 5). The severity of this defect, showed that the p51 subunit of the heterodimer does not catalyze viral DNA synthesis *in vivo*. Moreover, when the equivalent catalytic site mutation was analyzed in p51 (p51^{YMNN}/p66), virus infectivity was reduced to approximately 70% of that of p51/p66 (wild-type) complemented virus (Fig 6A, lane 4). Similarly, viral DNA synthesis of virus containing the p51^{YMNN}/p66 RT was also modestly

reduced compared to that of wild-type (Figure 6B and C, lanes 6). This showed that the YMDD aspartates of p51 affect viral DNA synthesis.

To further analyze the role of these p51 aspartates, they were mutated to alanines, glutamates, or lysines. Virus stocks containing each mutant RT were prepared by
5 cotransfection and analyzed for infectivity and DNA synthesis. Similar to the asparagine mutations, the glutamic acid mutations (p51^{YMEE}/p66) decreased virus infectivity and DNA synthesis only slightly (Figure 6). More dramatic decreases in both DNA synthesis and virus infectivity were observed for viruses containing either the alanine (p51^{YMAA}/p66) or the lysine (p51^{YMKK}/p66) p51 mutations. The effect of each of the p51 YMDD mutants on viral
10 DNA synthesis was examined using primer pairs that detect either early (R-U5) or late (R-gag) products of reverse transcription. The magnitude of the defect was similar with both primer pairs, showing that the defect was at or prior to initiation. The cellular expression of Vpr-p51 and p66 by these *trans* Vpr-RT constructs was equivalent, ruling out expression defects as the cause for differences in viral infectivity and DNA synthesis. The effect of
15 each of these mutations on DNA synthesis and infectivity correlate with the disruptiveness of the mutation introduced. This shows that the YMDD motif of p51, specifically its aspartate residues, is important to maintain the structure of the RT heterodimer and its enzymatic function *in vivo*.

Effect of expressing p66 and IN in cis

20 The results indicate the rescue of M7 infectivity to a maximal level of approximately 15% compared to wildtype SG3. This can be explained, at least in part, by reports showing defects in virions lacking RT-IN expression and packaging as a contiguous protein, included aberrant morphology and RNA conformation. In an attempt to enhance the complementation efficiency of the assay, a vpr-p51/p66-IN expression plasmid was constructed and
25 cotransfected into 293T cells with M7. Progeny virions exhibited decreased infectivity (lane 4) compared to virions generated with vpr-p51/p66. Virions concentrated by ultracentrifugation were analyzed by immunoblotting using the p66 (RNase H) specific MAb. The vpr-p51/p66 complemented virions (Fig. 18B, lane 3) incorporated p66 at levels comparable to wildtype SG3 (lane 1), while the RT-minus M7 virions showed no p66 (lane
30 2). Virions generated by cotransfection of M7 and vpr-p51/p66-IN (lane 4) had reduced p66 compared to vpr-p51/p66-derived virions (lane 3), and also showed a relatively large

amount of unprocessed p66-IN (RT-IN). Probing a replica blot with MAb to CA confirmed that approximately the same amount of each virus was analyzed (Fig. 18C).

Effect of expressing p66 and vpr-p51 from separated plasmids

The efficiency of complementation when the Vpr-p51 and p66 subunits were
5 expressed from separate mRNA in the transfected cells was examined. 293T cells were
cotransfected with M7, pLR2P-vpr-p51 (vpr-51), pLR2P-p66 (p66) and vpr-IN and progeny
virions were analyzed. Infectivity was rescued to levels similar to that exhibited previously
using vpr-p51/p66, about 10-13% of wildtype SG3. Complementation analysis using either
vpr-p51 or p66 only rescued infectivity by 0.1% and 0.2% of wildtype SG3, respectively
10 (lanes 2 and 3). These results indicate rescue of M7 virion infectivity when p66 and Vpr-p51
are coexpressed from separate mRNAs. Expression of Vpr-p51 and p66 from separate
genetic elements facilitates the manipulation of this approach for analyzing RT function,
since this allows the ratios of the two plasmids to be varied.

Distinction between Vpr-p51 and p66 on immunoblots

15 The vpr-p51/p66 construct places vpr and RT in-frame, and preserves the N-terminal
protease cleavage site of RT by including 11 amino acids of PR (11Pro) between Vpr and
RT. Thus, the molecular mass of the unprocessed Vpr-p51 fusion protein is
indistinguishable from that of p66 when analyzed by Western blotting. This has necessitated
the use of a MAb specific to the RNase H domain for specific detection of p66 in virions.
20 To distinguish between these two proteins (Vpr-p51 and p66) by molecular mass, PR
sequence encoding 30, 45 or 60 amino acids was introduced between the Vpr and p51
coding regions in vpr-p51 (vpr-^{30Pro}p51/p66, vpr-^{45Pro}p51/p66 and vpr-^{60Pro}p51/p66,
respectively). Immunoblot analysis of virions derived by cotransfection of vpr-^{30Pro}p51/p66
along with M7 into 293T cells showed that adding 30 amino acids of PR was not sufficient
25 to clearly differentiate Vpr-p51 and p66 (Fig. 19, lane 5). However, the addition of 45 or 60
PR residues allowed clear distinction between Vpr-p51 and p66 (lanes 6 and 7). Analysis of
infectivity for the 30Pro and 45Pro-derived trans-RT containing virions indicated that they
rescued M7 infectivity at levels comparable to the original (11Pro containing) vpr-p51/p66.
In contrast, the 60Pro rescued infectivity less efficiently (lane 7). Taken together, these
30 results indicate that vpr-^{45Pro}p51/p66 is a viable alternative to the original vpr-p51/p66
construct for analyzing trans-RT heterodimer structure/function. Specific detection of Vpr-
p51 and p66 based on molecular mass can facilitate quantitative analyses of the heterodimer.

Chemotherapeutic inhibition of the trans-RT heterodimer

The trans-heterodimer assay is of clinical relevance for analyzing HIV-1 RT inhibitors, drug resistance and the effects of drug resistance mutations on viral fitness. To examine the response of the trans-heterodimeric RT to anti-RT drugs, transfection-derived
5 M7 virions complemented with wildtype trans-RT (Vpr-p51/p66) were used to infect the TZM-bl indicator cells in the absence or presence of either 3TC (0.04, 0.2 and 1.0 μ M) or nevirapine (1.0, 5.0 and 5.0 μ M). Both drugs exerted a potent, dosage-dependent antiviral effect, as evidenced by an inhibition of infectivity. The IC₅₀s for 3TC and nevirapine were 0.138 and 0.011 mM, respectively. These results indicate that the effects of both NRTIs and
10 NNRTIs on the trans-heterodimeric RT are similar to those observed for RT derived from the Gag-Pol precursor of HIV-1 provirus (Fig. 20).

Discussion

Vpr-p51 and p66 form an intracellular dimer (Vpr-p51/p66) that is specifically incorporated into virions, processed by the viral PR to liberate p51/p66, and rescues the
15 infectivity of RT-deficient HIV-1. By analyzing mutations in the YMDD aspartates of either p51 or p66 the function of these residues in the context of an infectious virus was delineated. The absence of minus-strand strong-stop DNA synthesis in cells infected with virus, in which the YMDD aspartates of p66 were mutated, corroborates findings from previous *in vitro* studies, and demonstrates that in a heterodimer, p66 is solely responsible
20 for the catalytic/polymerase function of RT *in vivo*. The analysis of the p51 subunit indicates that mutations in the YMDD aspartates impair virus infection and DNA synthesis due to an effect on RT structure rather than catalytic function.

The YXDD motif (SEQ ID NO: 9) of retroviral RTs is highly conserved and it has been described in the active site of many viral and cellular polymerases (Kamer and Argos,
25 1984; Toh et al., 1983). The HIV-1 YMDD motif is situated in the palm domain (Ding et al., 1998; Kohlstaedt et al., 1992; Sarafianos et al., 2001). The Y183 and M184 amino acid residues contribute to the dNTP binding pocket of p66 (Huang et al., 1998). While some substitutions of these residues are tolerated, most mutations at these sites reduce polymerase function (Lowe et al., 1991; Wakefield et al., 1992). The most conserved feature of the
30 YMDD motif is the aspartates (D185 and D186), which together with a third aspartate (D110) form the polymerase catalytic triad. Mutation of the aspartates abolishes RT catalytic function and virus infectivity (Boyer et al., 1992; Larder et al., 1987b; Lowe et al.,

1991). The role of the catalytic aspartates in each RT subunit has been studied by expressing p51 and p66 separately in *Escherichia coli* (Hostomsky et al., 1992; Le Grice et al., 1991). Recombinant heterodimers containing polymerase active site mutations exclusive to p51 retain almost wild-type levels of polymerase activity, whereas heterodimers containing the same mutation(s) in p66 appear to be defective in polymerase activity. In the RT heterodimer, the polymerase domain of p51 assumes a closed conformation (Kohlstaedt et al., 1992), and therefore p51 does not appear to play a catalytic role in reverse transcription *in vivo*.

The role of the p51 YMDD aspartates in reverse transcription was investigated by analyzing the effects that different mutations had on reverse transcription and virus infectivity. The p51 YMDD aspartates were substituted with both conservative and non-conservative amino acid residues. The YMNN mutant is relatively conservative, since asparagine is almost isosteric to, but less charged than, Asp. In the YMEE mutant the length of the side chain is increased by one methylene group without changing the negative charge. Similar to aspartate, asparagine and glutamine are capable of participating in hydrogen bond interactions through their side chains. These two p51 mutants caused a slight reduction of infectivity and DNA synthesis. Substitution of the p51 YMDD aspartates with either alanines (YMAA) or lysines (YMKK) drastically reduced infectivity and DNA synthesis. The alanines have a short hydrophobic side chain that cannot make hydrogen bonds with neighboring polar residues. The lysines present an opposite polarity through a lengthened side chain. Small changes in charge and/or length of the side-chain can be tolerated (i.e. YMNN and YMEE), however, a charge shift and/or substantial changes in side chain length are not (i.e. YMKK and YMAA). These findings show that side chain interactions of p51 YMDD aspartates are important for RT function.

The D185 and D186 residues of p51 YMDD are within interacting distance (approximately 3 Å) of residues T409 and W410 of the p51 connection subdomain. The T409 and W410 residues lie in the loop between alpha helix L (α L) and beta sheet 20 (β 20). This loop is a part of the putative "tryptophan motif" (Trp-motif) of the p51 connection subdomain, which is believed to be critical for p51-p66 dimerization (Baillon et al., 1991; Divita et al., 1994; Tachedjian et al., 2003). It is plausible that mutation at the p51 YMDD aspartates cause repositioning of the α L- β 20 loop, which in turn could affect multiple interactions involving the Trp-motif and the heterodimer interface. The orientation of the

α L- β 20 loop could also influence template binding as these residues are in the proximity of the floor of the DNA binding cleft/RNase H primer grip, which includes K390, K395 and E396 of p51 that interact directly with the template-primer (Huang et al., 1998; Sarafianos et al., 2001). Mutation of the p51 YMDD aspartates may also affect intermolecular interactions that maintain its structure in the RT heterodimer. In the p51 subunit, the connection subdomain folds into the expanded cleft between its fingers and thumb subdomains, which gives it a "closed" conformation. Since the connection subdomain of p51 makes multiple contacts with the three other subdomains of p51 (fingers, palm and thumb), destabilization of the interaction between the α L- β 20 loop and YMDD can have global effects on RT folding. In addition to the interactions with T409 and W410, the YMDD motif is buried within the core of p51, and thus, the aspartates could interact with other neighboring residues. These include interdomain interactions between D185/186 and R72 of the p51 fingers subdomain; D185 and Q151-G152 at the tip of α helix E in the palm subdomain and D185/D186 with the tryptophan-rich region of p51 (Figure 7).

The finding that subunit-specific analysis of RT function can be studied using infectious virus has broad implications. While the p51 subunit was believed to function primarily as a scaffold to maintain the active structure of p66 (Hughes, 2001; Telesnitsky and Goff, 1997), other functions have been suggested, including involvement in tRNA primer-binding (Arts et al., 1994; Jacques et al., 1994), loading of p66 onto the template-primer (Harris et al., 1998) and enhancement of strand displacement (Amacker et al., 1995; Hottiger et al., 1994). The dimer interface between p51 and p66 is critical for reverse transcription and it has been proposed as an ideal target for therapeutic intervention (Divita et al., 1994; Morris et al., 1999; Restle et al., 1990). This notion was supported by several studies demonstrating that mutation of amino acid residues involved in subunit interactions alter the arrangement of the RT subdomains and disrupt RT function (Ghosh et al., 1996; Menendez-Arias et al., 2001; Tachedjian et al., 2003). Mutations in p51 have been also implicated in resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs) and inhibitors of RNase H activity. The E138K mutation, which confers resistance to TSAO {2',5'-Bis-O-(tert-butyl dimethylsilyl)-3'spiro-5''-(4''-amino-1'',2''-oxathiole-2'',2''-dioxide)} has been mapped to the p51 subunit (Jonckheere et al., 1994; Sluis-Cremer et al., 2000). The C280S mutation in RT causes resistance to the RNase H inhibitor N-ethylmaleimide (NEM) (Loya et al., 1997). Both the p51 and p66 subunits were found to contribute to the resistance of the enzyme to NEM *in vitro*.

Materials and Methods

Cells and Antibodies

The 293T, JC53 (Platt et al., 1998), and TZM-bl cell lines (Wei et al., 2002) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplement with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). The anti-RT antiserum (R1465) was generated against HIV-1 RT expressed in *E. coli*. Briefly, the entire RT coding region of HIV-1/pSG3 was ligated into the prokaryotic pGEX expression vector (pGEX-RT). *E. coli* (DH5 α) were transformed with pGEX-RT and protein expression was induced with isopropyl β -D-thiogalactopyranoside (IPTG). Expression of the glutathione S-transferase- (gst) RT fusion protein was confirmed by SDS-PAGE. Soluble gst-RT protein was purified and RT was released by thrombin cleavage using previously described procedures (Smith and Johnson, 1988). New Zealand white female rabbits were immunized subcutaneously with 200 μ g of purified RT protein emulsified in an equal volume of Freund's complete adjuvant. Rabbits were boosted at two week intervals with 200 μ g of RT mixed with an equal volume of Freund's incomplete adjuvant. Sera were tittered and analyzed for specificity by immunoblotting against purified preparations of both the immunizing protein and concentrated HIV-1 virions. Additional antibodies used in these studies included monoclonal antibodies to HIV-1 capsid (183-H12-5C) and HIV-1 RT (8C4 and 7E5).

HIV-1 proviral clones

The HIV-1 pSG3 proviral clone (SG3) (Ghosh et al., 1993) (Genbank Accession # L02317) was used to produce wild-type virus, and to construct RT deficient proviral clones and all recombinant RT and IN expression plasmids. The pSG3^{FN} (FN) clone was constructed using the strategy described by Dubay *et. al.* (Dubay et al., 1992) for the HXB2 pFN clone (Figure 1B). Briefly, the FN clone contains an in-frame 110 amino acids deletion and was created by *Acc65I* digestion to remove a 330-nucleotide fragment of the *pol* gene. The 5' overhang was filled using dGTP and the Klenow fragment of DNA polymerase. The remaining single-stranded regions were removed with S1 nuclease and the plasmid was religated. The deleted DNA segment encoded a large part of RNase H and 13 amino acids of the carboxyl-end of the polymerase domain of RT. This clone encodes a truncated form of RT while maintaining the IN coding region in-frame.

The pSG3^{M7} (M7) proviral construct was created from pSG3^{S-RT} (S-RT) (Wu et al., 1997). In addition to stop codons in the RT and IN coding regions of pSG3^{S-RT}, M7 contains three additional stop codons at amino acid positions 441 (TAA), 444 (TGA) and 447 (TAG) and a D443N RNase H catalytic mutation in the RNase H reading frame. The primers (sense [5'-AAGCCCGGGATGGATGGCCCAAAGT-3'], SEQ ID NO: 10 and antisense [5'-TCCTAAACGCGTCTCCCTCTAAGCTGCTCAATTTACTTAGAAAGT-3'], SEQ ID NO: 11) containing *Xma*I and *Mlu*I sites, respectively, and the primers (sense [5'-ACTTTCTAAGTAAATTGAGCAGCTTAGAGGGAGACGCGTTTAGGA-3'] (SEQ ID NO: 12) and antisense [5'-TATGTCGACACCCAATTATGAAAAG-3'] (SEQ ID NO: 13)) containing *Mlu*I and *Sal*I sites, respectively, were used to amplify two DNA fragments from the S-RT constructs (nucleotides 2132-3455 and 3410-5340). The *Xma*I-*Mlu*I and *Mlu*I-*Sal*I PCR products were digested with corresponding restriction endonucleases, purified and ligated together into an *Xma*I-*Sal*I cut pSG3^{S-RT} plasmid.

Construction of heterodimeric RT expression plasmid

To express the RT subunits in trans with RT-minus proviral DNA, the pLR2P-vpr-p51-IRES-p66 (*vpr-p51/p66*) expression plasmid was constructed. Briefly, the sense [5'-TAGATCAGATCTGTTGACTCAGATTGGTTGCA-3'] (SEQ ID NO: 14) and antisense [5'-ATCTACACGCGTTTAGAAGGTTTCTGCGCCTT-3'] (SEQ ID NO: 15) primers containing the *Bgl*II and *Mlu*I restriction sites (underlined), respectively, were used to PCR amplify a p51-containing DNA fragment from pSG3. The internal ribosome entry site (IRES) was PCR amplified from the encephalomyocarditis virus (EMCV) (Duke et al., 1992) (Genbank Accession # NC_001479) using the sense ([5'-TTATTAACGCGTCCGCCCTCTCCCTCCCCC-3'] (SEQ ID NO: 16) and antisense [5'-CCATCCCGGGCTTTAATTTTACTGGTACAGTTTCAATAGGAC TAATGGGTCCCATGGTATTATCGTCTT-3'] (SEQ ID NO: 17) primers containing *Mlu*I and *Xma*I sites (underlined), respectively. The PCR-derived p51 fragment was digested with *Bgl*II and *Mlu*I, while the IRES fragment was digested with *Mlu*I and *Xma*I. These two fragments were ligated simultaneously into the *Bgl*II-*Xma*I-cut pLR2P-vprRT (Wu et al., 1997), generating pLR2P-vpr-p51-IRES-p66. This construction strategy (Figure 1A) placed *vpr* and *RT* in-frame, while preserving the N-terminal protease cleavage (PC) site of RT by including 33 bps of PR sequence 5' of RT. The antisense primer introduced a translational stop codon (TAA) to terminate RT expression at amino acids 440, which is the full-length

p51 subunit. The vpr-p51 reading frame was followed by the IRES and then p66. To enable efficient expression of p66, an artificial Kozak sequence was included at the 5' of the p66 coding sequence (Kozak, 1987). This modification added a Met-Gly onto the p66 N-terminus. The pLR2P-vpr- Δ p51-IRES-p66 (*vpr- Δ p51/p66*) control plasmid was constructed to contain a translational stop codon at the first amino acid position of p51 by amplification of a *Bgl*II-*Mlu*I DNA fragment from the S-RT clone. Other derivatives of *vpr-p51/p66* were constructed using PCR based site-directed mutagenesis, restriction digestion with the appropriate enzyme, and cloning into the *Bgl*II-*Mlu*I or *Xma*I-*Xho*I sites for p51 or p66 mutant clones, respectively. All clones were confirmed by sequencing. The pLR2P-vprIN (*vpr-IN*) expression vector has been described previously (Wu et al., 1997).

Transfections and analysis of virus infectivity

DNA transfections were performed on monolayer cultures of 293T cells grown in 6-well plates using the calcium phosphate DNA precipitation method. Unless otherwise noted, each cell monolayer (well) was transfected with 6 μ g of proviral DNA, 3 μ g of the *vpr-p51/p66* constructs and 1 μ g of the *vpr-IN* constructs. Culture supernatants from the 293T cells were collected 60 h post-transfection, clarified by low-speed centrifugation (1000 x g, 10 min), and filtered through 0.45 μ m pore-size sterile filters. The clarified supernatants were analyzed for HIV-1 p24 antigen concentration by ELISA (Beckman-Coulter Inc.).

Virus infectivity was assessed using the TZM-bl reporter cell line as described earlier (Wei et al., 2002). Briefly, virus containing supernatants were normalized for p24 antigen concentration, serially diluted (five-fold dilutions) and used to infect monolayer cultures of TZM-bl cells. At 48 hrs post-infection, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) reagent as described earlier (Kimpton and Emerman, 1992). The blue-stained cells were counted using a light microscope. Wells containing between 30 and 300 blue cells were used to calculate the infectious units of virus per ng of p24 antigen (IU/p24-ng).

Semiquantitative detection of viral DNA

The PCR method used to analyze the synthesis of nascent viral DNA in infected cells was similar to those described earlier (Fassati and Goff, 2001; Zack et al., 1990). Briefly, 500-ng equivalents (p24 antigen) of transfection-derived virions were incubated with DNase I (4 µg/ml; Worthington Inc.) at 37°C for 1 hr to minimize plasmid DNA carryover. The treated virus was then used to infect one million JC53 cells for 4 hrs in DMEM (1% FBS, 10 µg/ml DEAE-dextran). The cells were washed twice with DMEM, and the medium was replaced with complete DMEM (10% FBS). At 18 hrs post-infection, the cells were lysed and total DNA was extracted by organic methods, resuspended in 200 µl of distilled water and treated with the *DpnI* restriction endonuclease to digest bacterially derived plasmid DNA. Each PCR subjected 250 pgs of DNA extract to 30 rounds of amplification with primers designed to detect early (R-U5 [sense nucleotides 79-99 AGCTTGCCCTGAGTGCTTCAA (SEQ ID NO: 18) and antisense nucleotides 182-157 CTGCTAGAGATTTTCCACACTGACTA] SEQ ID NO: 19) and late (R-gag [sense nucleotides 43-63 GGCTAGCTAGGGAACCCACTG (SEQ ID NO: 20) and antisense nucleotides 355-334 ATACTGACGCTCTCGCACCCAT] (SEQ ID NO: 21)) viral DNA. The PCR products were separated on a 1.0% agarose gel and visualized by ethidium bromide staining. The relative amount of amplified DNA was determined by comparison to known standards (serial dilutions of pSG3 DNA).

Western Blot (immunoblot) analysis

Transfection-derived virions were concentrated by ultracentrifugation through 20% sucrose cushion (125,000 x g, 2 hr, 4°C) using a SW41 rotor (Beckman Inc.). Pellets were solubilized in loading buffer (62.5 mM Tris-HCl [pH 6.8], 0.2% SDS, 5% 2-mercaptoethanol, 10% glycerol), boiled, and proteins were separated on 12.0% polyacrylamide gels containing SDS. Following electrophoresis, proteins were transferred to nitrocellulose (0.2-µm pore size) by electroblotting and incubated for 1 hr at room temperature in blocking buffer (5% nonfat dry milk in phosphate-buffered saline [PBS]). The blocked blot was exposed to the appropriate primary antibody for 1 hr in blocking buffer with constant mixing. After extensive washing, bound antibodies were detected by chemiluminescence using horseradish peroxidase-conjugated species-specific secondary antibodies (Southern Biotechnology Associates, Inc.) as described by the manufacturer (Amersham Biosciences).

Example 2: Exogenous Reverse Transcriptase Assay

Disclosed are biochemical assays for determining reverse transcriptase activity. One example of such an assay is the Chemiluminescent Reverse Transcriptase Assay by Roche (Cat. No. 1 828 657, Instruction Manual Version 3, February 2004, herein incorporated by reference in its entirety for its teaching regarding reverse transcriptase assays). The protocol is a non-radioactive enzyme immunoassay useful for highly sensitive, quantitative determination of reverse transcriptase activity by chemiluminescence detection. This assay is designed for highly-sensitive and quantitative determination of RT activity, e.g. in cell cultures and other life science research applications. The assay has been shown to be useful for the determination of RT activity derived from a variety of retroviruses, including HIV-1, HIV-2, SIV-1 and CAEV. The assay can be used to determine the propagation of retroviruses in retrovirus-infected mammalian cell cultures. The assay can also be used for *in vitro* screening for RT inhibitors.

Example 3: p51-IRES-p66 Rescues the Infectivity of RT-IN-Minus Virus (M7)

Regarding Table 4: the table shows that Vpr-p51-ires-p66 rescues the infectivity of RT-IN-minus virus (M7), and viruses derived from proviral DNA containing mutations in the YMDD motif of RT, including YMAA and YMND. Virus derived from proviral DNA and the control pLR2P-vpr is not infectious. Methods: 293T cells were transfected with the indicated plasmid (either viral DNA or trans-RT DNA) DNAs plus the pLR2P-vor-IN expression plasmid DNA. 48 hrs later the supernatant viruses were collected and analyzed for infectivity using the JC53-BL reporter assay.

Table 4:

Construct Name		Blue Cells		Infectious virions/ml	p24 ngs/ml	Virions/ng	% of SG3
		A		$A/B \times 1000 = C$	D	C/D	
pLR2P-vpr	--	93		#DIV/0!	714	#DIV/0!	100.00
pLR2P-vpr	--	0		0.00E+00	397	0.00E+00	0.00
pLR2P-vpr	--	0		#DIV/0!	688	#DIV/0!	#DIV/0!
vpr-p51-IRES-p66	--	66		#DIV/0!	563	#DIV/0!	#DIV/0!
pLR2P-vpr	--	0		#DIV/0!	382	#DIV/0!	#DIV/0!
vpr-p51-IRES-p66	--	21		#DIV/0!	1072	#DIV/0!	#DIV/0!

Construct Name		Blue Cells	Dilution	Virions/ml	p24 ngs/ml	Virions/ng	% of SG3
		A	B	$A/B \times 1000 = C$	D	C/D	
pLR2P-vpr	--	225	0.2	1.13E+06	714	1.58E+03	100.00
pLR2P-vpr	--	0	5	0.00E+00	397	0.00E+00	0.00
pLR2P-vpr	--	2	5	4.00E+02	688	5.81E-01	0.04
vpr-p51-IRES-p66	--	843	5	1.69E+05	563	2.99E+02	19.01
pLR2P-vpr	--	0	5	0.00E+00	382	0.00E+00	0.00
vpr-p51-IRES-p66	--	66	5	1.32E+04	1072	1.23E+01	0.78

5 **Example 4: The Tryptophan Motif of HIV-1 Reverse Transcriptase Structural analysis of the putative RT dimerization domain (tryptophan motif)**

The Trp-motif of HIV-1 is comprised of aromatic amino acids in the connection subdomain (between amino acid positions 398 and 14). Alignment analysis shows that the six tryptophans (W398, W401, W402, W406, W410 and W414) and tyrosine (Y405)

10 residues are conserved within the connection subdomain of most primate lentiviruses (Figure 1A). The most conserved residue amongst all the lentiviruses is W398. To understand the interactions at the dimerization interface between the two connection subdomains of HIV-1 RT, several crystal structures of HIV-1 RT, including unliganded (1DLO), were compared in complex with substrates (pdb codes 1TO5, 1RTD, 1HYS,

15 1N6Q) or NNRTIs (1HNI, 1SV5, 1S6P, 1S9E, 1DTT, 1BMQ, 1FK9). The overall structure of the dimerization interface is conserved among the various RT complexes. Stabilization of the heterodimer involves direct, as well as indirect interactions between residues from the

two subunits. Specifically, a key direct interaction appears to involve three p51 residues from the β 18- α K (N363) loop, the α L helix (W401), and the α L- β 20 loop (Y405), that are within interacting distance of residue W410 located in the α L- β 20 loop of p66 (Figure 10). In addition to these interactions, the W401 in p51 is also within interacting distance with p66 residue P412 at the base of the β 20-sheet in p66. Indirect interactions can also play a role and involve residues that are proximal to the p66 or the p51 part of the interface. In the p51 subunit, a cluster of four Trp residues (W398, W402, W406 and W414) is proximal to the p51 interface residues (Y405, N363, and W401) (Figure 10).

Expression and virion incorporation of heteromeric RT containing p51 Trp-motif mutations

The p51 Trp-motif residues were independently mutated to alanines in the pLR2P-vpr-p51-IRES-p66 (*vpr-p51/p66*) expression plasmid. Wildtype and each of the mutant DNAs were cotransfected into 293T cells with the RT-IN defective M7 proviral DNA, and progeny virions were examined by immunoblot analysis. The control RT-IN-minus M7 particles (Figure 2A, lane 2) did not contain RT. A similar level of both RT subunits (p51 and p66) was detected in particles derived by cotransfection of M7 and the wildtype *vpr-p51/p66* expression plasmid (lane 3). The pLR2P-vpr- Δ p51-IRES-p66 (*vpr- Δ p51/p66*) control plasmid does not express p51, and the absence of detectable p66 (lane 4) confirmed that its incorporation was dependent on the expression of p51 (Vpr-p51). Analysis of p51^{W398A}/p66 (lane 5) showed p51 and p66 in the virion, however, an additional band was also detected migrating just below p66. This band was confirmed to be a product of the p66 subunit by probing with mAb (7E5) specific to the RNase H domain. To a lesser extent, a similar p66 product was also seen in some of the other p51 mutants. Notably, the aberrant p66 product seemed to associate with mutants of residues (W398A, W402A, W406A and W414A) that cluster together proximal to the heterodimer interface (Figure 10C). Wildtype HIV-1 SG3 virions were analyzed as an additional control (lane 1). Immunoblot analysis using mAb to CA confirmed that approximately the same amount of each virus was analyzed (Figure 11B). Examination of transfected cells by immunoblotting with polyclonal anti-Vpr serum (Figure 11C) demonstrated that all of the mutants expressed Vpr-p51 (lanes 5-11) at levels similar to that of wildtype Vpr-p51 (lane 3). A replica blot probed with 7E5 mAb confirmed that p66 was expressed at a similar level among the transfected cells (Figure 11D). The amount of cellular protein analyzed was similar, as demonstrated by probing for the α -tubulin protein (Figure 11E).

To examine if the aberrant p66 was due to misprocessing by the viral protease (PR), 293T cells were cotransfected with a PR-defective proviral DNA (PR catalytic mutant: D25A) and *vpr-p51^{W398A}/p66*. Detection of the aberrant p66 product in these virions (data not shown), suggested that it was generated independently of the HIV-1 PR.

5 *Functional analysis of p51 Trp-motif RT mutants*

The functionality of the p51 Trp-motif mutants was analyzed in a single-round infectivity assay, using the TZM-bl reporter cell line (Wei et al. *Antimicrob. Agents Chemother.* 46:1895-905 (2002)). Virions were generated by cotransfecting 293T cells with M7, wildtype or mutant *vpr-p51/p66* and *vpr-IN*. *vpr-IN* was included since M7 lacks IN, which is required for efficient initiation of reverse transcription and for integration of the nascent viral cDNA (Mulky et al. (2004); Wu et al. *J. Virol.* 73:2126-35 (1999)). The infectivity of virions containing the wildtype *trans-RT* (Vpr-p51/p66) was normalized to 100% (Figure 11F, lane 1). The infectivity of M7 derived by cotransfection with *vpr-Δp51/p66* was less than 0.2% compared to *vpr-p51/p66* (lane 2). Mutations in the tryptophan cluster (W398A, W402A, W406A and W414A) decreased infectivity to less than 50% (lanes 3, 5, 7 and 9, respectively), with the W398A mutant being the most defective. The infectivity of the p51^{W401A}/p66 mutant (lane 4) was similar to that of wildtype, while the p51^{Y405A}/p66 mutant (lane 6) was reduced to about 50%. The p51^{W410A}/p66 mutant (lane 8) had little effect on infectivity.

20 *Subunit-specific mutagenesis of Trp-motif residues at the heterodimer interface*

The analysis of inter-subunit interactions was focused initially on mutagenesis of individual residues to either alanine or leucine. The infectivity of the wildtype RT *trans*-heterodimer was normalized to 100% (Figure 12A, lane 1). The *vpr-Δp51/p66* was less than 0.2% infectious (lane 2). Replacement of p51^{W401} with either alanine or leucine did not affect viral infectivity (lanes 3 and 4). Both the p51^{Y405A}/p66 and p51^{Y405L}/p66 mutant RTs reduced infectivity to approximately half of that of the wildtype *trans*-heterodimer (lanes 5 and 6, respectively). Mutation of N363 in p51 to alanine also reduced infectivity, albeit to a lesser extent than the 405 mutations (lane 7). The replacement of p66^{W410} with alanine caused a slight reduction in infectivity (lane 8), while the leucine substitution had no effect (lane 9). The p51/p66^{L234A} and p51/p66^{W401A} mutants, reported previously as mutations that affect dimer formation, were included as controls in the experiments (Tachedjian et al.

(2003), Ghosh et al. *Biochemistry* 35:8553-62 (1996)). The p51/p66^{L234A} mutant reduced infectivity to less than 5% (lane 10), while the p51/p66^{W401A} mutant was approximately 40% infectious (lane 11).

To further delineate these Trp-motif interactions, residues that lie within interacting distance of each other were mutated in pairs. Mutations were made in conjunction at residues W401 and W410 of p51 and p66, respectively. Infectivity analysis of p51^{W401A}/p66^{W410A}, p51^{W401A}/p66^{W410L} and p51^{W401L}/p66^{W410A} showing that mutagenesis of both residues together reduced viral infectivity (approximately 40%) to a significantly greater extent compared to that of the single mutations (Figure 12B, lanes 1, 2 and 3). Analysis of RT containing simultaneous mutations at p51^{Y405} and p66^{W410} indicated that substitution of both residues with alanine decreased infectivity to about 25% of the wildtype heterodimer (lane 4). In contrast, the infectivity of the p51^{Y405A}/p66^{W410L} double mutant (lane 5) was comparable to that of the p51^{Y405A}/p66 single mutant, showing that mutagenesis of p66^{W410} to leucine does not affect its interaction with Y405 of p51. The model predicted that the residue N363 in p51 interacts with both p51^{Y405} and p66^{W410}. The p51^{N363A;Y405A}/p66 (lane 6) and p51^{N363A}/p66^{W410A} (lane 7) virions had similar infectivity, which was reduced to approximately 35% of wildtype and substantially lower than the respective single mutants. Immunoblot analysis detected only a slight reduction in Vpr-p51-mediated p66 incorporation in some of the double mutants.

Analysis of the inter-subunit interface in provirus

The results indicated an interaction at the dimer interface between residues p51^{W401} and p66^{W410} that is important for subunit interaction. Additional analysis of the W401A and W410A mutations was conducted in the context of the complete HIV-1 NL4-3 proviral clone. The wildtype or mutant proviral DNAs were transfected into 293T cells and progeny virions were analyzed for infectivity. The infectivity of virus containing the W401A mutation was less than 5% of that of wildtype (Figure 4A, lanes 1 and 3). In contrast, W410A caused an increase in virus infectivity (lane 4). The non-infectious RT-minus M7 clone was included as negative control (lane 2). Notably, immunoblot analysis showed a significantly reduced amount of the W401 mutant RT in virions, compared to either the wildtype or W410A mutant (Figure 13B). Probing a replica blot with mAb to CA confirmed that approximately the same amount of each virus was analyzed (Figure 13C).

Analysis of W401A mutation in the RT trans-heterodimer

To determine the effect of the W401A proviral DNA mutation on RT, the mutation was analyzed by subunit-specific *trans*-complementation, wherein the mutation was present p51, p66 or both p51 and p66. The infectivity of virions complemented with the wildtype
5 *trans*-heterodimeric RT was normalized to 100% (Figure 5A, lane 1). Subunit-specific mutagenesis of p51 (p51^{W401A}/p66) did not significantly affect viral infectivity, as described above (lane 3), while mutagenesis of the p66 subunit (p51/p66^{W401A}) reduced infectivity to about 40% (lane 4). The effect of this mutation in both subunits (p51^{W401A}/p66^{W401A}) was quite dramatic (lane 5), reducing infectivity to levels similar to that observed for the W401A
10 mutant provirus.

Analysis of virions produced by coexpressing *vpr-p51^{W401A}/p66* demonstrated, as expected, wildtype levels of both subunits (Figure 5B, lanes 1 and 3). However, when the W401A mutation was present in the p66 subunit (*vpr-p51/p66^{W401A}*), the incorporation of p66 into M7 virions was reduced (lane 4). Interestingly, the presence of W401A in both p51
15 and p66 further reduced the amount of p66 detected in virions (lane 5). In all cases, reduced virion p66 was also seen using a polyclonal RT antiserum and the amount of p66 expressed in the cells was normal (data not shown). The decrease in virion p66 observed with the p51/p66^{W401A} and p51^{W401A}/p66^{W401A} mutants was identical to that observed when virions were produced using a PR-defective proviral DNA (PR catalytic mutant: D25A) in place of
20 M7. This result confirmed that less p66 was detected in the M7 virions because the W401A mutation(s) impaired p66 virion incorporation. Importantly, this result ruled out the possibility that less p66 was detected due to overprocessing and conversion of p66 to p51 by the viral protease subsequent to virion assembly.

To analyze the infectivity of *trans*-RT complemented virions in a target cells that is
25 more physiologically relevant, the JLTRG-R5 reporter cell line was used. These cells are derived from JLTRG cells and are of Jurkat T cell lineage. (Ochsenbauer-Jambor et al. *submitted* (2004); Kutsch et al. *Antimicrob. Agents Chemother.* 48:1652-63 (2004)). The JLTRG-R5, cells have stably integrated EGFP reporter under control of the HIV-1 LTR, and thus EGFP expression is induced by virus infection. Infectivity for the wildtype *trans*-RT
30 heterodimer was normalized to 100% (Figure 14C, lane 1). The Δp51/p66 exhibited infectivity below 5% (lane 2). The W401A mutation in p51 did not affect viral infectivity (lane 3), while W401A in p66 reduced infectivity to about 50% of that of the wildtype

trans-RT (lane 4). The presence of W401A simultaneously in p51 and p66 significantly decreased infectivity (lane 5). These results were consistent with those generated using the TZM-bl assay, which was used for analysis in a parallel experiment. The ability to analyze virions containing *trans*-heteromeric RT using a T cell line emphasizes the biological relevance of our approach. The results indicate that the *trans*-RT heterodimer complemented virions can be analyzed in multiple human-derived reporter cell lines including more physiologically relevant T cell lines.

Efavirenz enhances subunit interaction in the trans-RT W401A double mutant

To examine the effect of dimerization enhancing drugs on the dimerization-defective W401A mutant *trans*-heterodimeric RT, EFV was added to the producer cells (transfected 293T cells) at concentrations ranging from 0.01-1.0 μ M. Examination of virion-associated p66^{W401A} incorporation, which is dependent upon interaction with p51^{W401A} (Vpr-p51^{W401A}), showed that EFV rescued subunit dimerization in a dose-dependent manner (Figure 15A). Equal virion protein loading was confirmed by probing a replica blot with anti-CA mAb (Figure 15B). The amount of virion-associated p51 and Vpr-p51 was equal in both the absence and presence of drug. Similar results were also observed for other second generation NNRTIs.

Discussion

Two significant problems have heretofore hindered structure/function studies of RT using infectious virus. First, RT is encoded and assembled into virions as part of the Prl60^{Gag-Pol} polyprotein, and consequently, mutations in RT/Prl60^{Gag-Pol} can be pleiotropic, affecting multiple steps of the viral life cycle such as assembly, maturation, etc. (Yu et al. *Virology* 219:29-36 (1996), Quillent et al. *Virology* 219:29-36 (1996), Olivares et al. (2004), Tomonaga et al. *Arch. Virol.* 143, 1-14 (1998)). Analogous with the results for the W401A proviral mutant, Yu et al. have reported that mutations in the polymerase primer grip decrease virion-associated RT due in part to premature Gag-Pol processing (Yu et al. *Virology* 219:29-36 (1996)). The second problem was due to the heterodimeric nature of the RT. The asymmetry of the p51 and p66 subdomains entails that a mutation in one subunit is structurally and functionally non-equivalent to the same mutation in the other subunit. Thus a novel *trans*-complementation approach for analyzing the RT heterodimer in precise was developed molecular detail in the context of infectious virions. By exploiting

this approach, several relevant questions concerning HIV-1 RT biology have been answered that were previously experimentally inaccessible. Primarily, these include (i) the role of hydrophobic, amino acid residues comprising the Trp-motif for subunit interaction and RT function, (ii) the contribution of amino acids at the p51/p66 connection subdomain interface to RT dimerization and virus infection, and (iii) the availability of a biologic approach capable of assessing the effects of both dimerization enhancing and disrupting drugs.

Structural analysis of interactions in the Trp-motif with residues at the subunit interface in several complexes of RT with substrate or inhibitors shows that the side-chain of W410 in the p66 α L- β 20 loop, consistently within interacting distance of p51 residues W401, Y405, and N363 (Figure 1D). *trans*-complementation analysis of these putative inter-subunit interactions showed that mutation of individual residues at this interface caused a measurable decrease in virus infectivity. Simultaneous mutagenesis of two inter-subunit residues within interacting distance of each other further impaired viral infectivity, showing that this effect was due to effects on subunit interactions. The data from immunoblot indicate similar to wildtype (Vpr-p51/p66) levels and processing of the mutant trans-RT of the two subunits.

The most severe effect on heterodimerization was observed for the p51^{W401A}/p66^{W401A} mutant. The presence of W401A in both subunits markedly impaired p51-p66 interaction, directly evidenced by a significant decrease in Vpr-p51^{W401A} mediated p66^{W401A} packaging. Based on this data, the structural analysis of several RT crystal structures and previous reports on the Trp-motif, repositioning the α L- β 20 loop by mutating p66^{W401} and disruption of interactions involving p51^{W401}, p51^{W405}, p51^{N363} and p66^{W401} account for the findings. Subunit-specific mutational analysis of the W401 RT mutants demonstrates that W401 of the p66 and p51 subunits has distinct structural roles in the stabilization the RT heterodimer. In p51 the W401A mutation appears to affect the interactions at the interface, through disruption of the π - π interactions with p66^{W410}. However, the p66-W401A mutation affects the folding of the p66 subunit because it is at the interface of two helices (α L and α K) (Figure 1C). Hence, when both subunits are mutated the different effects appear additive.

Structural analysis of the dimer interface in several RT crystal structures highlighted the potential importance of a cluster of four tryptophans in p51 (W398, W402, W406 and W414) proximal to the dimer interface. While these four p51 tryptophans do not directly

interact with p66 residues, they are clustered together through hydrophobic interactions and seem poised to indirectly affect the dimer interface by their proximity to residues Y405, W401, and N363 of p51 that are at the p51-p66 interface (Figures 10B and 10C). Subunit-specific mutagenesis of these residues suggests that the Trp cluster affects the interaction
5 between p51 and p66 (Figure 11). Alanine substitution resulted in a misprocessed form of p66 that was detected in virions, *trans*-complementation analysis using PR defective virus indicates that a cellular protease is responsible for the aberrant processing of p66. The p51 Trp mutants can interact with and incorporate into virions a smaller processed form of p66 generated in the cell. Failure to detect the aberrant p66 in cell lysates, suggests it is present
10 at a significantly lower level than wildtype p66. Alternatively, these p51 Trp mutants might form unstable heterodimers in which p66 is misfolded and thus, susceptible to proteolytic processing by a cellular protease. If this were true, it is interesting to note that dimer instability causes p66 misprocessing instead of normal processing to generate p51, which can associate with disassociated p66 to give functional RT heterodimer. The defect in
15 infectivity seen with the mutants containing misprocessed p66 further supports this interpretation. Although p66 misprocessing seems to occur as a consequence of the atypical manner in which RT is expressed via the *trans*-complementation approach, it appears that residues W398, W402, W406 and W414 are important for proper RT subunit interactions.

Example 5: Materials and Methods

20 *Cells, antibodies and antiviral drugs*

The 293T, JC53 (Platt et al. *J. Virol.* 72:2855-64 (1998), and TZM-bl cell lines (Wei et al. *Antimicrob. Agents Chemother.* 46:1895-905 (2002) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). The JLTRG-R5 cell line 34 was maintained in
25 Roswell Park Memorial Institute (RPMI) 1640 medium containing 15% FBS and gentamycin (0.1 mg/ml). Antibodies used included polyclonal anti-RT and anti-Vpr sera (Wu et al. *J. Virol.* 69:389-98 (1995) and mAbs to human α -tubulin (Sigma), HIV-1 CA (183-H12-5C) and HIV-1 RT and RNase H (8C4 and 7E5), respectively, obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Plasmid constructs

The HIV-1 pSG3 (SG3) proviral clone (Genbank Accession # L02317) (Ghosh et al. *Virology* 194:858-64 (1993)) was used to produce wildtype virus, and to construct the RT-deficient proviral clone and all recombinant RT and IN expression plasmids (for abbreviations of plasmids see Table 3). The pSG3^{FN} (FN) clone was constructed using the strategy described by Dubay et al. (J. Virol. 66:6616-25, 1992) for the HXB2 pFN clone (Fig. 1b). Briefly, the FN clone contains an in-frame 110 amino acids deletion and was created by Acc65I digestion to remove a 330-nucleotide fragment of the pol gene. The 5' overhang was filled using dGTP and the Klenow fragment of DNA polymerase. The remaining single-stranded regions were removed with S1 nuclease and the plasmid was ligated. The deleted DNA segment encoded a large part of RNase H and 13 amino acids of the carboxyl-end of the polymerase domain of RT. This clone encodes a truncated form of RT while maintaining the IN coding region in-frame. The RT-IN-minus pSG3^{M7} (M7) proviral construct was used for trans-complementation analysis with all the pLR2P-based RT and IN expression plasmids. For expressing the RT subunits in *trans*, the pLR2P-vpr-p51-IRES-p66 (vpr-p51/p66) plasmid 31 was modified by including 135 bp of PR sequence 5' of RT. This increased the molecular weight of the Vpr-RT fusion protein and enabled visual separation from p66 by immunoblot analysis. Briefly, p51-encoding sequence was PCR amplified from SG3 using primers containing BglII and MluI restriction sites, respectively. The internal ribosome entry site (IRES) was PCR amplified from the encephalomyocarditis virus (EMCV) (Genbank Accession # NC_001479) with primers containing MluI and XmaI sites, respectively. The p51 and IRES DNA fragments were digested with corresponding endonucleases and ligated simultaneously into the BglII-XmaI cut pLR2P-vprRT 37. The vpr-p51/p66 was modified in that the N-terminal protease cleavage (PC) site of RT was maintained by including 135 bps of PR-encoding sequence 5' of RT compared to 33 bps of PR sequence in the original construct. The vpr and p51 coding sequences were placed in-frame, with a translational stop codon (TAA) to terminate RT expression at amino acids 440, which is the full-length p51 subunit. The vpr-p51 reading frame was followed by the IRES and then the p66-encoding DNA sequence. Mutant derivatives of vpr-p51/p66 (Table III) were constructed using PCR-based site-directed mutagenesis and cloning into the BglII-MluI or XmaI-XhoI sites for p51- and p66-containing DNA fragments, respectively. The pLR2P-vpr-Δp51-IRES-p66 (vpr-Δp51/p66) control expression plasmid was constructed to contain a translational stop codon at the first

amino acid position of p51 (Mulky et al. (2004)). This plasmid controls for non-specific incorporation of p66 into viral particles. All mutant clones were confirmed by nucleotide sequence analysis. The pLR2P-vprIN(vpr-IN) expression vector has been described previously (Wu et al. *EMBOJ.* 16:5113-22 (1997)).

5 *Construction of heterodimeric RT expression plasmids.*

The pLR2P-vpr-p51-IRES-p66 (vpr-p51/p66) plasmid was constructed for independent expression of the RT subunits in trans. Since the molecular mass of the unprocessed Vpr-p51 fusion protein is very similar to that of p66, these two proteins are not distinguishable using antibody directed to the polymerase domain of RT. To allow
10 differentiation between Vpr-p51 and p66 by molecular mass, three derivatives of the original *vpr-p51/p66* construct were made. These constructs were generated by including additional PR sequence 5' of the p51-coding region in vpr-p51. Either 90, 120 or 150 bps of PR sequence (encoding 30, 45 and 60 amino acids, respectively) were introduced at this position, generating *vpr*^{-30Pro}*p51/p66*, *vpr*^{-45Pro}*p51/p66* and *vpr*^{-60Pro}*p51/p66*, respectively.
15 The *vpr-p51/p66-IN* was constructed by cutting the pLR2P-vpr-RT-IN plasmid with XmaI-XhoI and ligating the RT-IN fragment with XmaI-XhoI cut *pLR2P-vpr-p51-IRES-p66*. The *vpr-Δp51/p66* control plasmid was constructed to contain a translational stop codon at the first amino acid position of p51. Other derivatives of *vpr-p51/p66* were constructed using PCR-based site-directed mutagenesis and cloning into the BglII-MluI or XmaI-XhoI sites of
20 either p51 or p66, respectively. All clones were confirmed by nucleotide sequencing.

Transfection and analysis of virus infectivity

DNA transfections were performed on monolayer cultures of 293T cells grown in 6-well plates using the calcium phosphate DNA precipitation method. Unless otherwise noted, each cell monolayer (well) was transfected with 6 µg of proviral DNA, 3 µg of the
25 *vpr-p51/p66* constructs and 1 µg of the *vpr-IN* construct. Culture supernatants from the 293T cells were collected 60 h post-transfection, clarified by low-speed centrifugation (1000 x g, 10 min), and filtered through 0.45 µm pore-size sterile filters. The clarified supernatants were analyzed for HIV-1 p24 concentration by ELISA (Beckman-Coulter Inc.).

Virus infectivity was assessed using the TZM-bl reporter cell line as described
30 earlier (Wei et al. *Antimicrob. Agents Chemother.* 46:1895-905 (2002)). Briefly, virus containing supernatants were normalized for p24 antigen concentration, serially diluted

(five-fold dilutions) and used to infect monolayer cultures of TZM-bl cells. At 48 h post-infection, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl- β -D-galacto X-gal staining, the blue-stained cells were counted using a light microscope. Wells containing between 30 and 300 blue cells were used to calculate the infectious units of virus per ng of p24 antigen (IU/p24-ng).

The ability of *trans*-RT-containing virions to infect T cells was tested by quantitatively analyzing infection of the JLTRG-R5 reporter T cell line. 12-well flat-bottomed culture plates containing 1.0×10^5 JLTRG-R5 cells were infected at a multiplicity of infection (MOI) of 5.0 (as determined by the TZM-bl assay) for the wildtype Vpr-p51/p66 complemented virions. The other *trans*-RT-containing virion preparations were normalized for p24 antigen equivalent to that of that of the wildtype *trans*-RT. The total volume was adjusted to 1 ml and the infection was carried at 37°C for 24 h. At 24 h post-infection, 1 ml of fresh RPMI 1640 was added to each well and culture was continued at 37°C for an additional 48 h. Then the cells were washed (2x) in phosphate buffered saline (PBS). The cell pellet was resuspended in 50 μ l PBS and then fixed in 1% paraformaldehyde (in PBS). The expression of EGFP was measured using a FACStar Plus flow cytometer with CellQuest software (Becton Dickinson).

Effects of NNRTIs on trans-RT subunit interaction

DNA transfections were performed on monolayer cultures of 293T cells grown in 6-well plates using the FuGENE 6 Transfection Reagent (Roche), as recommended by manufacturer. The M7 and *vpr-p51/p66*-based plasmids were used at a ratio of 2:1. At 24 h post-transfection, the specified concentrations of drug were added. Culture supernatants from the 293T cells were collected 60 h post-transfection, clarified by low-speed centrifugation (1000 x g, 10 min), and filtered through 0.45 μ m pore-size sterile filters. The clarified supernatants were processed for and analyzed by immunoblot as described below.

Immunoblot analysis

Transfection-derived virions were concentrated by ultracentrifugation through 20% sucrose cushion (125,000 x g, 2 hr, 4°C) using a SW41 rotor (Beckman Inc.). Pellets were solubilized in Laemmli loading buffer (62.5 mM Tris-HCl [ph 6.8], 0.2% SDS, 5% 2-mercaptoethanol, 10% glycerol), boiled, and proteins were separated on 12.0% polyacrylamide gels containing SDS. Following electrophoresis, proteins were transferred

to nitrocellulose (0.2- μ m pore size) by electroblotting and incubated for 1 h at room temperature in blocking buffer (5% nonfat dry milk in PBS). The blocked blots were exposed to an appropriate primary antibody for 1 h in blocking buffer with constant mixing. After extension washing, bound antibodies were detected by chemiluminescence using horseradish peroxidase-conjugated species-specific secondary antibodies (Southern Biotechnology Associates, Inc.).

Inhibition of trans-RT using NRTI and NNRTI

Virions were derived by cotransfection of 293T cells with M7, vpr-p51/p66 and vpr-IN. The TZM-bl cells were seeded overnight in 24-well plates at a concentration of 40,000 cells in 250 μ l of medium per well. The culture medium was removed and replaced with 250 μ l of DMEM containing 1% FBS and 2x drug concentrations (5-fold dilutions). 250 μ l of virus suspension normalized for equal IU, as determined by TZM-bl assay (diluted in DMEM containing 1% FBS and 80 μ g/ml DEAE-dextran), was then added to the cells. The two RT drugs used in this analysis, 3TC and nevirapine, were at final concentrations ranging from 0.04-1.0 μ M and 1.0-25.0 μ M, respectively. The cells were fixed 48 h post-infection, stained with X-gal reagent, and the blue-stained cells were counted using a light microscope as described above. The 50% inhibition concentration (IC₅₀) was measured with a 95% confidence interval.

Example 6: Trans-complementation analysis of Vpr-p51-IRES-p66

#	Viral DNA	trans-RT	Blue Cells	Dilution	Infectious virions/ml	p24 conc., ng/ml	Infectious virions/ng p24	% of SG3
			A	B	$A/B \times 1000 = C$	D	C/D	
1	pSG3-WT	pLR2P-vpr (cont)	93	0.2	4.65E+05	714	6.51E+02	100.00
2	SG3-M7	pLR2P-vpr (cont)	0	5	0.00E+00	397	0.00E+00	0.00
3	SG3-YMND	pLR2P-vpr (cont)	0	5	0.00E+00	688	0.00E+00	0.00
4	SG3-YMND	vpr-p51-IRES-p66	66	1	6.60E+04	563	1.17E+02	18.00
5	SG3-YMAA	pLR2P-vpr (cont)	0	5	0.00E+00	382	0.00E+00	0.00
6	SG3-YMAA	vpr-p51-IRES-p66	21	5	4.20E+03	1072	3.92E+00	0.60

20

The table shows the trans-complementation analysis of Vpr-p51-IRES-p66 with viruses derived from proviral DNA containing mutations in the YMDD motif of RT,

including YMAA and YMND. pLR2P-vpr is used as a negative control. The results show that the trans-heterodimeric RT can be expressed with proviral DNA, including mutant DNA, and incorporated into virions produced from the said cells, and function in reverse transcription. Methods: 293T cells were transfected with the indicated plasmid (either viral DNA or trans-RT DNA) DNAs. 48 hrs later the supernatant viruses were collected and analyzed for HIV-1 p24 antigen and infectivity using the JC53-BL reporter assay.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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and IN incorporated into HIV-1 particles independently of the Gag/Pol precursor protein. *EMBO J.*, **16**, 5113-5122.

Wu, X., Liu, H., Xiao, H., Kim, J., Seshiah, P., Natsoulis, G., Boeke, J.D., Hahn, B.H. and Kappes, J.C. (1995) Targeting foreign proteins to human immunodeficiency virus particles via fusion with Vpr and Vpx. *J. Virol.*, **69**, 3389-3398.

Yu, Q., Ottmann, M., Pechoux, C., Le Grice, S. & Dariix, J.-L. "Mutations in the Primer Grip of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Impair Proviral DNA Synthesis and Virion Maturation." *Virology* 219:29-36 (1996).

Zack, J.A., Arrigo, S.J., Weitsman, S.R., Go, A.S., Haislip, A. and Chen, I.S. (1990) HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell*, **61**, 213-222.

What is claimed is:

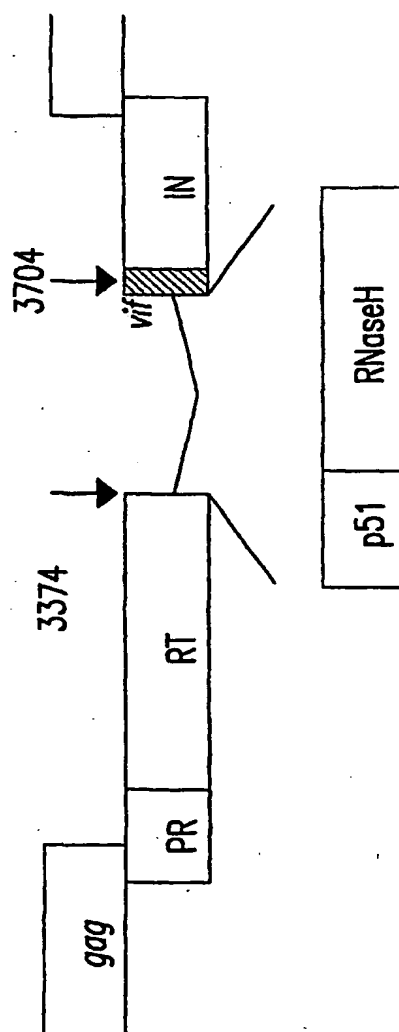
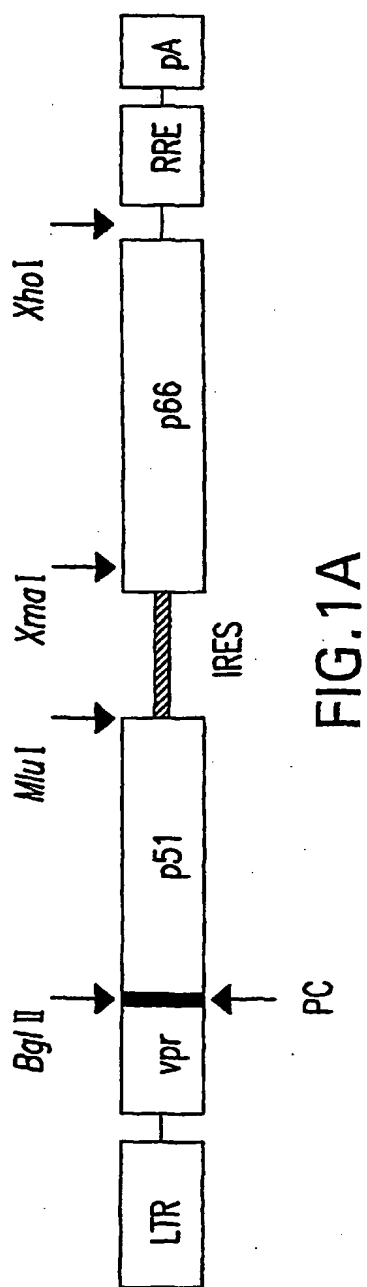
1. A cell comprising: (i) a vector comprising a p66 subunit, a p51 subunit, and Vpr, wherein Vpr and p51 are expressed as a fusion protein; (ii) and a retrovirus proviral DNA.
2. A method of screening for a compound that inhibits viral reverse transcriptase comprising: a) contacting the cell of claim 1 with the compound, and b) comparing the level of viral infectivity in the presence of the compound with the level of viral infectivity in the absence of the compound, wherein a decreased level of infectivity in the presence of the compound indicates that the compound inhibits reverse transcriptase.
3. A method of screening for a compound that inhibits viral reverse transcriptase comprising: a) contacting the cell of claim 1 with a compound; and b) comparing the level of p66 in virus particles generated by the cell.
4. A method of screening for a compound that inhibits viral reverse transcriptase comprising: a) contacting the cell of claim 1 with a compound; and b) comparing the level of reverse transcriptase in virus particles generated by the cell.
5. The method of any one of claims 2-4, wherein the virus is a lentivirus.
6. The method of claim 5, wherein the virus is HIV-1.
7. The method of any one of claims 2-4, wherein the p51 and p66 subunits are expressed in trans in the cell.
8. The method of any one of claims 2-4, wherein the p51 and p66 subunits are expressed on different messenger RNAs.
9. The method of any one of claims 2-4, wherein the p51 and p66 subunits are expressed on the same messenger RNAs.
10. The method of any one of claims 2-4, wherein expression of Vpr-p51 incorporates p66 protein into viral particles.
11. The method of any one of claims 1-4, wherein p51 interacts with p66 protein.
12. The method of any one of claims 1-4, wherein p51 contains a mutation, insertion, or deletion.
13. The method of any one of claims 1-4, wherein p66 contains a mutation, insertion, or deletion.

14. The method of any one of claims 1-4, wherein the plasmid also expresses an internal ribosome entry site (IRES).
15. A method of screening for a compound that affects dimerization of a p66 subunit polypeptide of reverse transcriptase and a p51 subunit polypeptide of reverse transcriptase comprising: a) contacting the cell of claim 1 with the compound, and b) comparing the level of complex formation in the presence of the compound with the level of complex formation in the absence of the compound, a change in the level of complex formation indicating that the compound affects dimerization of the p66 subunit and a p51 subunit.
16. A method of screening for a compound that inhibits dimerization of a p66 subunit polypeptide of reverse transcriptase and a p51 subunit polypeptide of reverse transcriptase comprising: a) contacting the cell of claim 1 with the compound, and b) comparing the level of complex formation in the presence of the compound with the level of complex formation in the absence of the compound, a lower level of complex formation indicating that the compound inhibits dimerization of the p66 subunit and a p51 subunit.
17. A method of screening for a compound that enhances dimerization of a p66 subunit polypeptide of reverse transcriptase and a p51 subunit polypeptide of reverse transcriptase comprising: a) contacting the cell of claim 1 with the compound, and b) comparing the level of complex formation in the presence of the compound with the level of complex formation in the absence of the compound, a higher level of complex formation indicating that the compound enhances dimerization of the p66 subunit and a p51 subunit.
18. A method of making a pharmaceutical composition which comprises: a) determining whether a compound inhibits reverse transcriptase by the method of claim 2; and b) admixing the compound with a pharmaceutically acceptable carrier.
19. A method of inhibiting viral reverse transcriptase comprising contacting (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of the compound identified by the method of claim 2, thereby inhibiting viral reverse transcriptase.

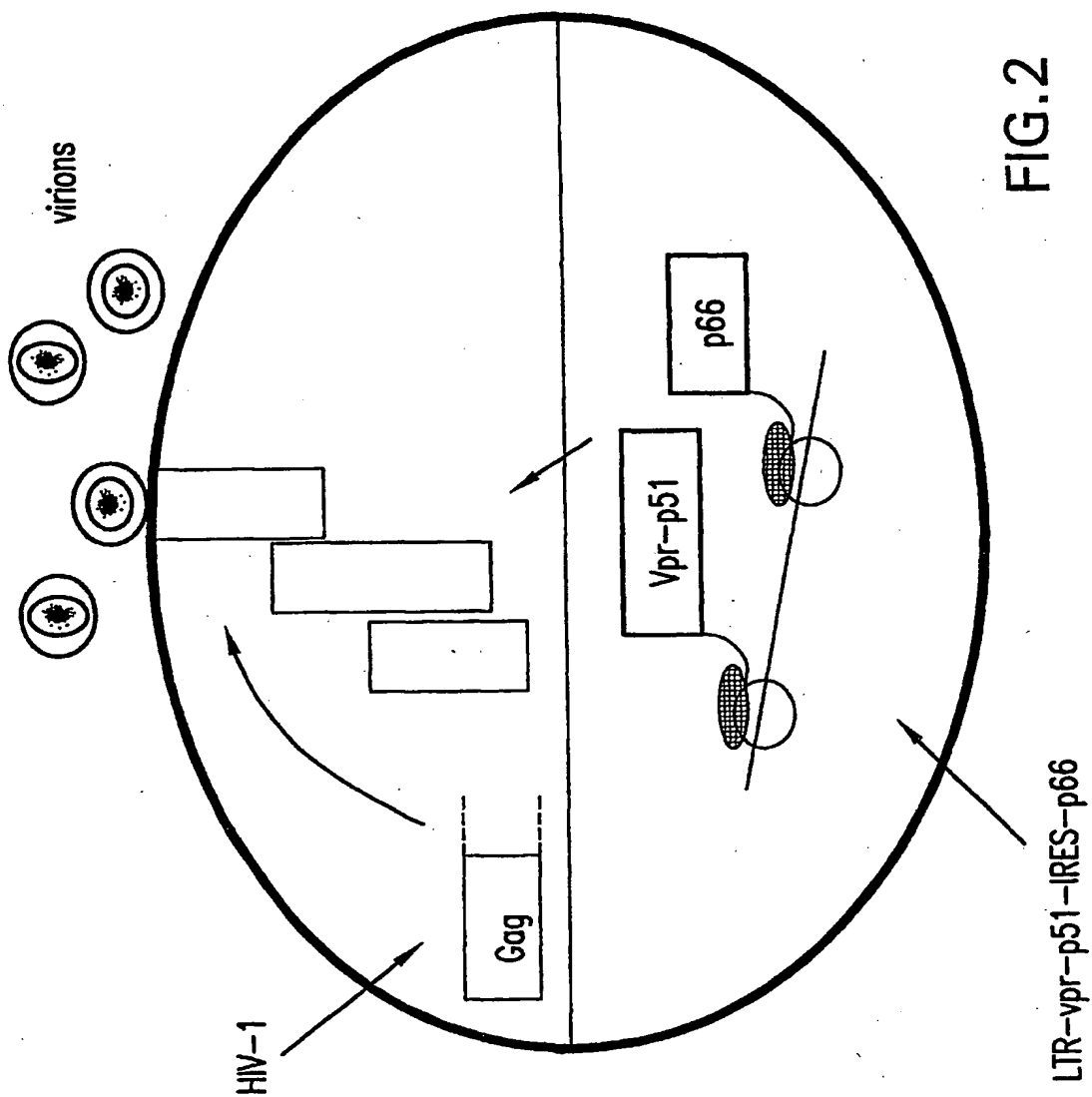
20. A method of inhibiting dimerization of a p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase, which comprises contacting either (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of the compound identified by the method of claim 16, thereby inhibiting dimerization of the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase.
21. A method of enhancing dimerization of a p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase, which comprises contacting either (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of the compound identified by the method of claim 17, thereby enhancing dimerization of the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase.
22. The method of claim 21, wherein the HIV-1 reverse transcriptase is present in a subject.
23. The method of claim 22, wherein the compound is administered to the subject orally, intravenously, subcutaneously, intramuscularly, topically or by liposome-mediated delivery.
24. A compound identified by the method of claim 2.
25. A compound identified by the method of claim 15.
26. A compound identified by the method of claim 16.
27. A composition which comprises the compound of claim 24 and a carrier.
28. The compound of claim 24, wherein the compound is capable of inhibiting HIV-1.
29. The compound of claim 28, wherein the compound is a nonnucleoside reverse transcriptase inhibitor.
30. An expression cassette comprising LTR-vpr-p51-IRES-p66.
31. The expression cassette of claim 30, wherein the nucleic acid comprises SEQ ID NO: 1.
32. The method of claim 2, wherein the HIV or SIV particles are derived by genes expressed in the cell and wherein the genes contain one or more nucleotide mutations.
33. A transgenic animal expressing vpr-p51/66.

34. A cell line comprising an exogenous nucleic acid, the nucleic acid comprising vpr-p51/66.
35. The cell line of claim 34, wherein the cell expresses viral nucleic acids.
36. The cell line of claim 34, wherein the cell can be induced to express viral nucleic acids by contacting the cell with a stimulus.

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FIG.3A



FIG.3B

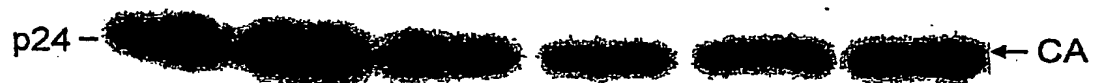


FIG.3C

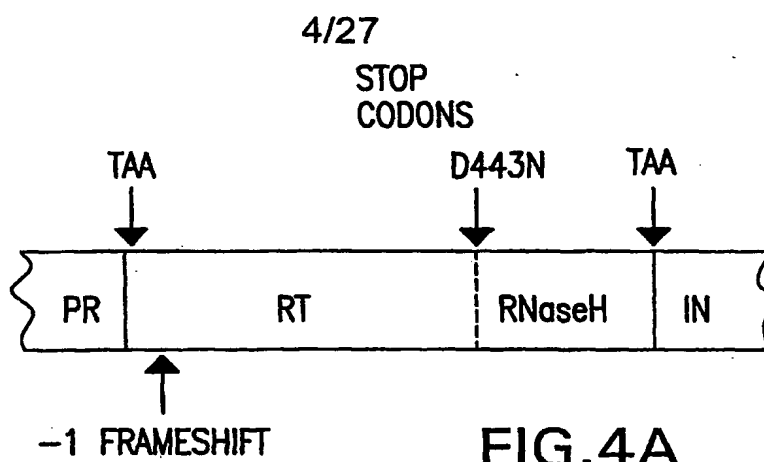


FIG.4A

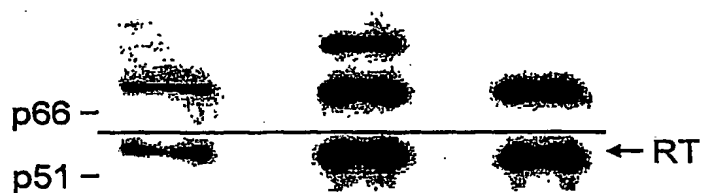


FIG.4B



FIG.4C

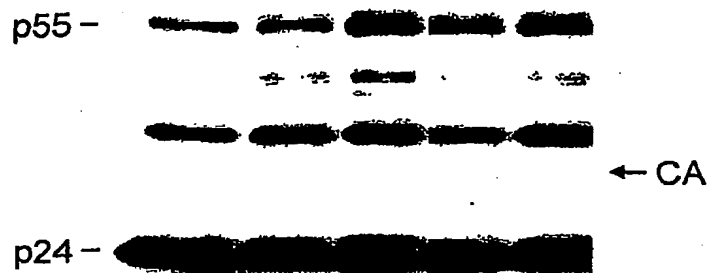


FIG.4D

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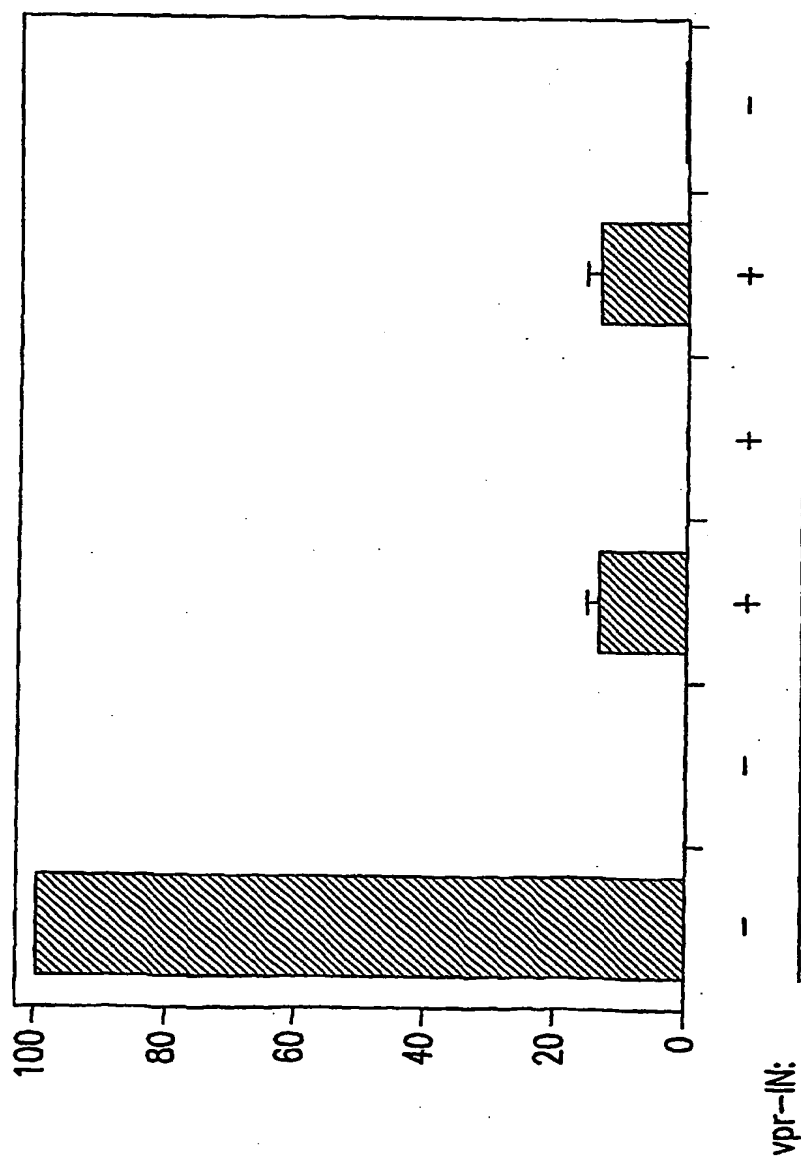


FIG. 5

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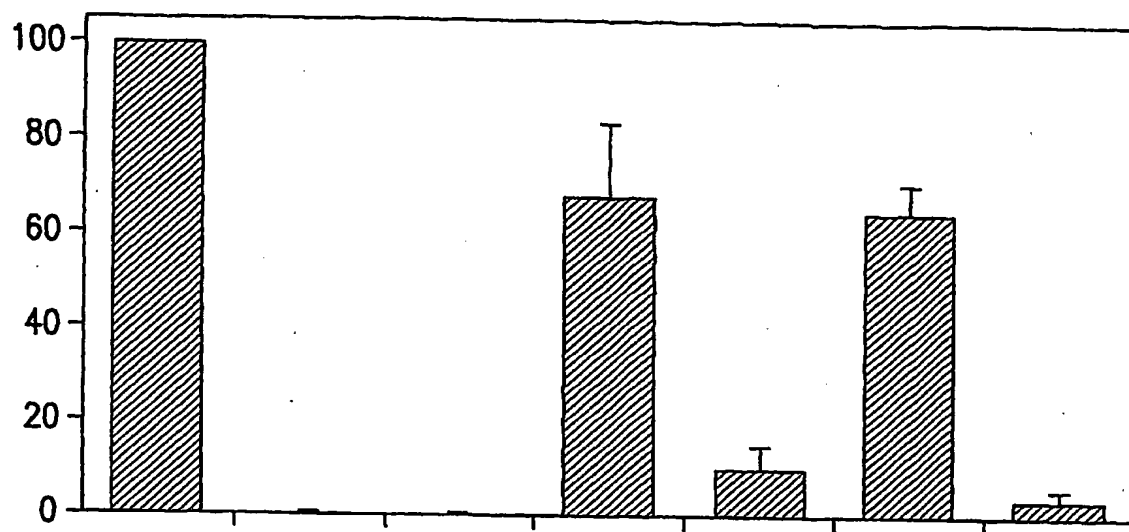
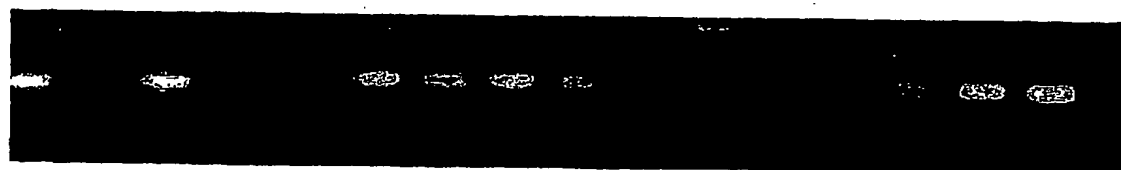


FIG.6A



copies of pSG3

FIG.6B



copies of pSG3

FIG.6C

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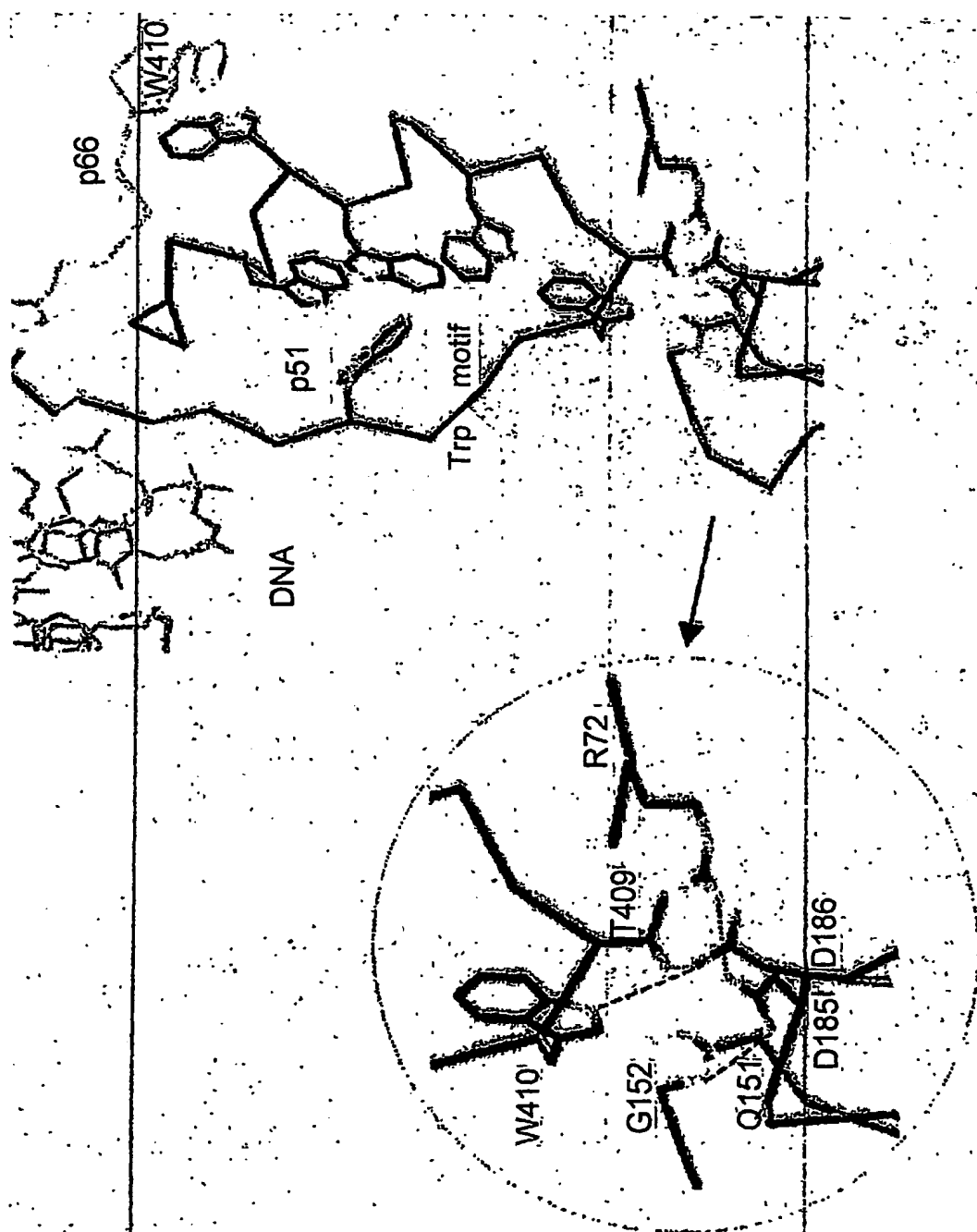


FIG.7

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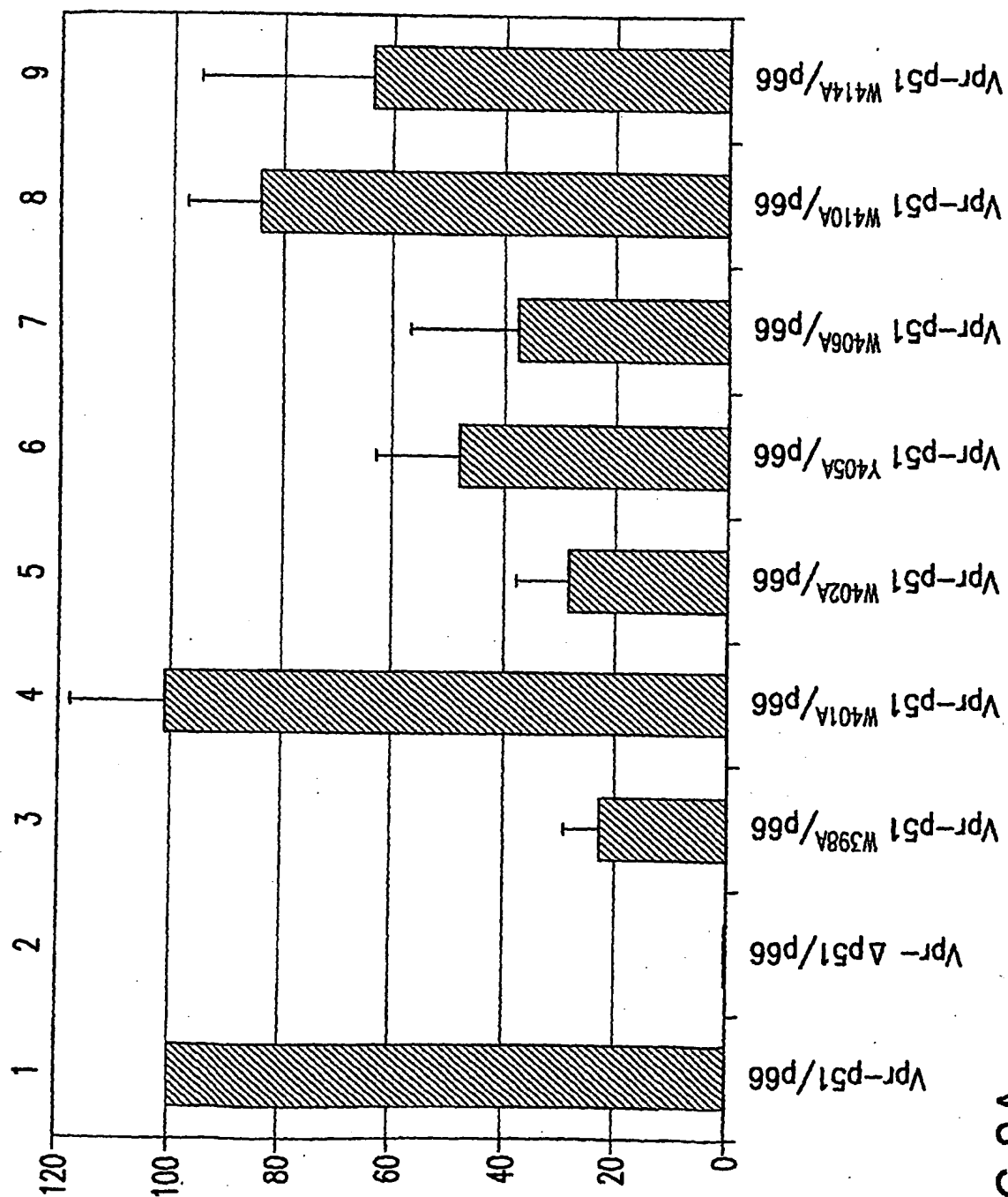


FIG.8A

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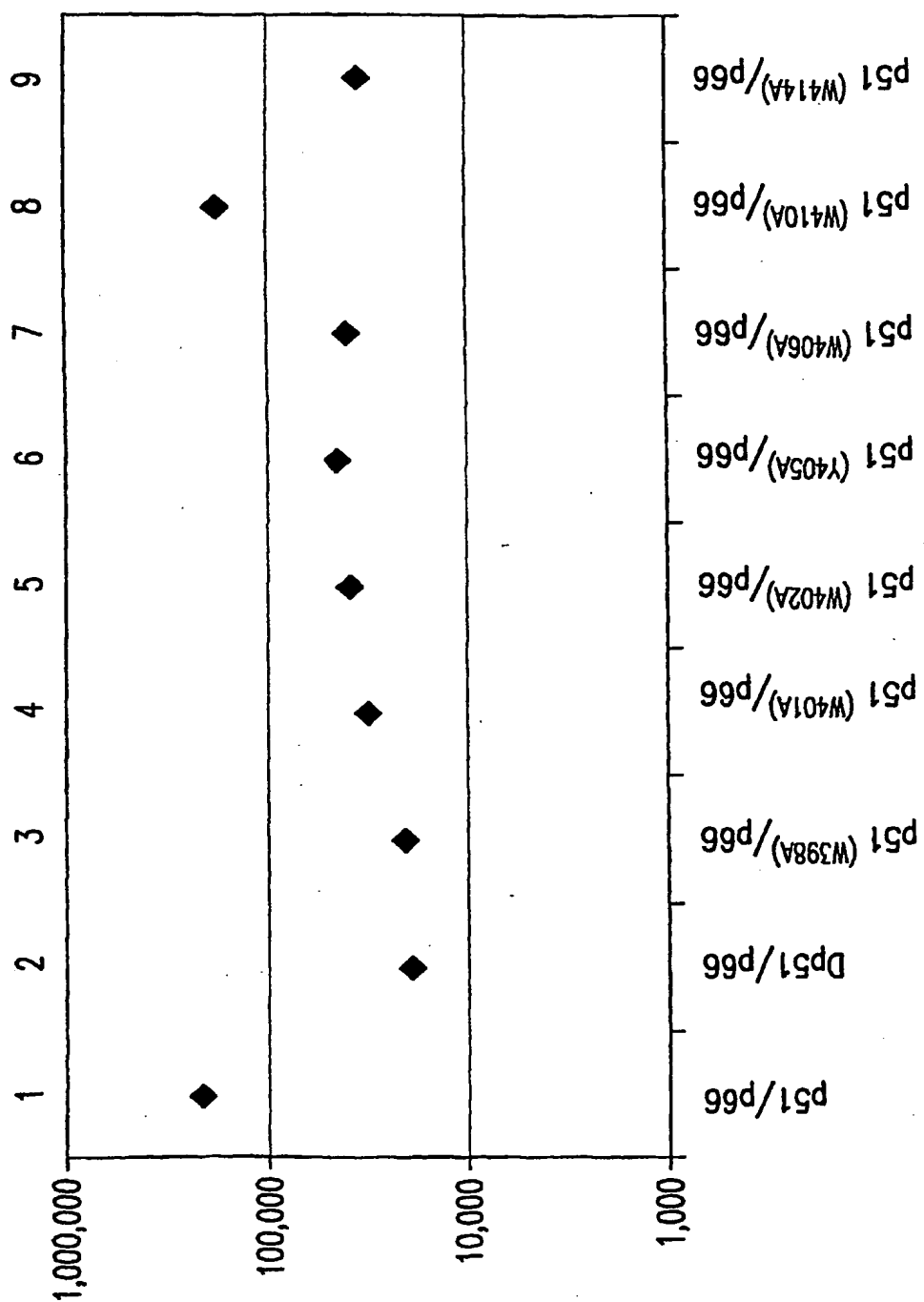


FIG. 8B

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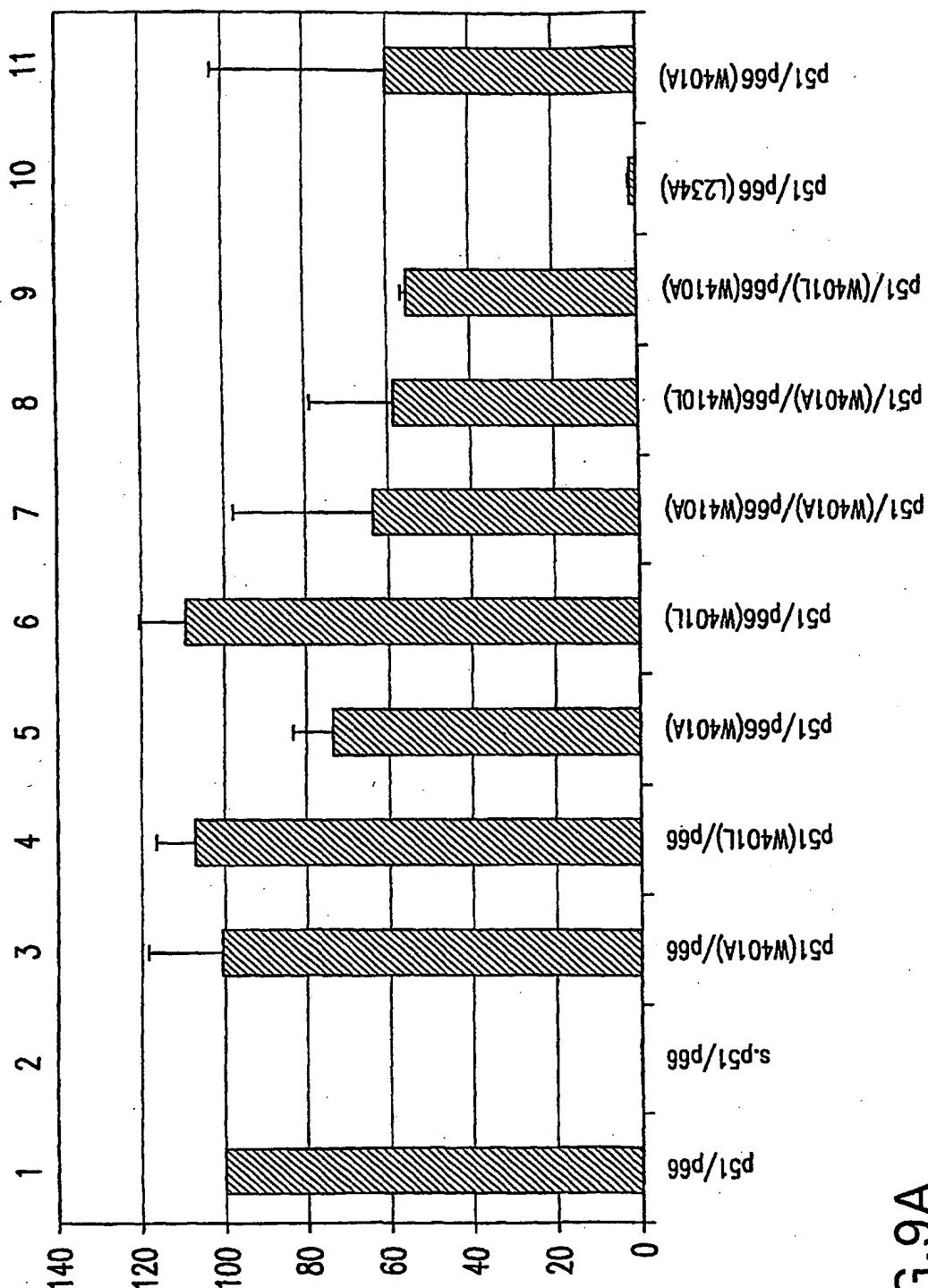


FIG. 9A

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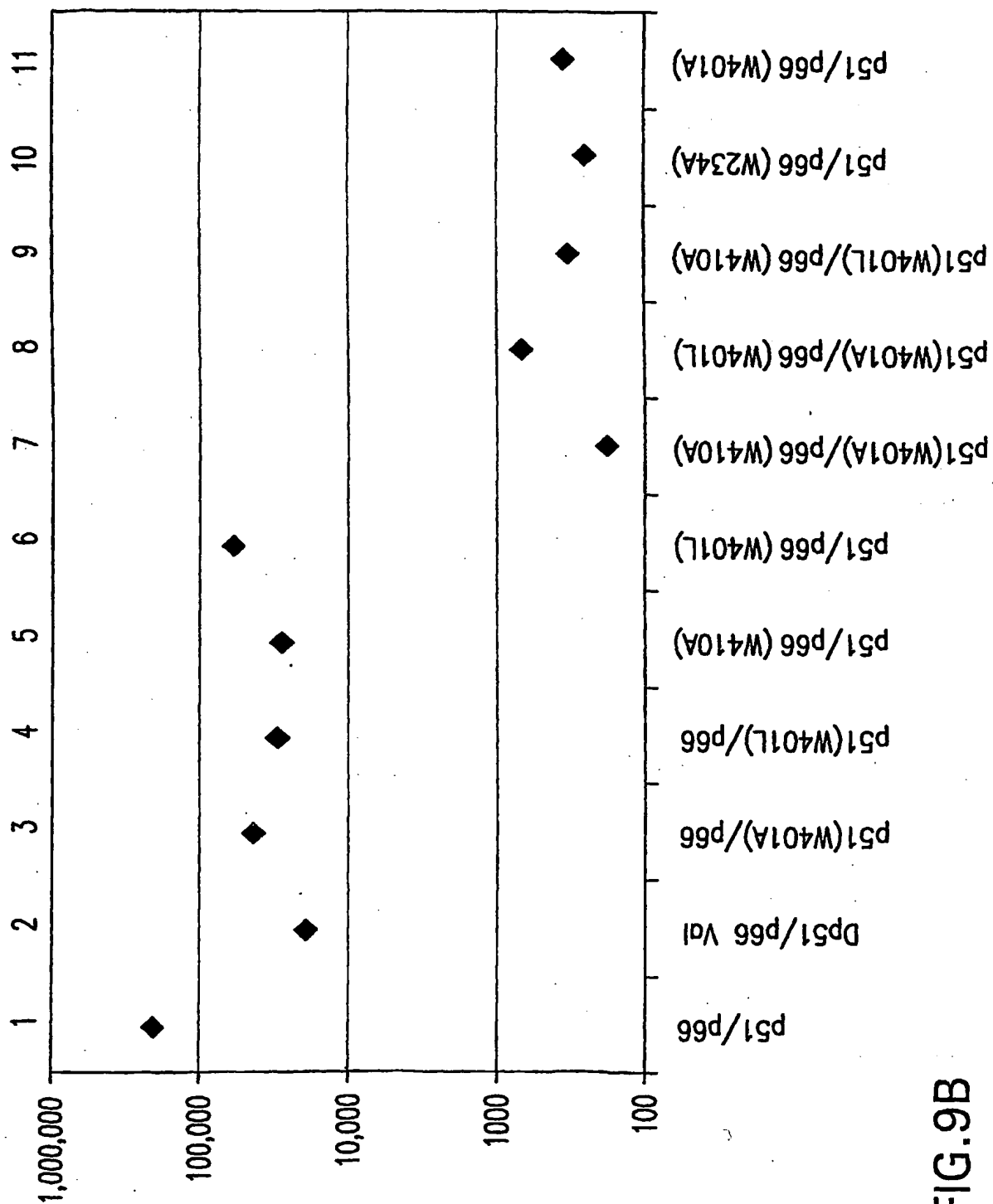


FIG. 9B

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	W398	W401	W402	Y405	Y406	Y410	Y414
HIV-1 ^{SG3} (L02317)	KETWETWTE..	YWQATWIPE.WE					
HIV-1 ^{NL4-3} (AF070521)	KETWEAWTE..	YWQATWIPE.WE					
HIV-2 ^{BEN} (M30502)	RETWEQWWDN..	YWQVTWIPE.WD					
HIV-2 ^{ROD} (M15390)	REIWEQWWDN..	YWQVTWIPD.WD					
SIV ^{cpz} (GAB1C) (X52154)	KESWEAWWAE..	YWQATWIPE.WE					
SIV ^{cpz} (TAN1) (AF447763)	KETWSQWWD..	YWQVTWVPE.WE					
SIV ^{AGM} (X07805)	REVWEQWWAD..	YWQVSWIPE.WD					
SIV ⁶⁷⁷ (agm) (M58410)	KKTWDMWWQD..	YWQVSWIPE.WE					
SIV ^{rcm} (AF349680)	REVWEQWWS..	YWQVSWIPE.WE					
SIV ^{syk} (L06042)	REEWEKWWD..	YWQATWVPE.VK					
SIV ^{col} (AF301156)	REDWEQWWS..	YWQSAWIPE.IE					
SIV ^{hoest} (AF188115)	REVWDQWWS..	HWQVTWIPE.LE					
SIV ^{sun} (AF131870)	REIWSQWWAD..	YWQCTWIPE.LE					
SIV ^{wrc} (AY138268)	KDIWDSWWS..	YWQVSWIPD.TE					

FIG.10A

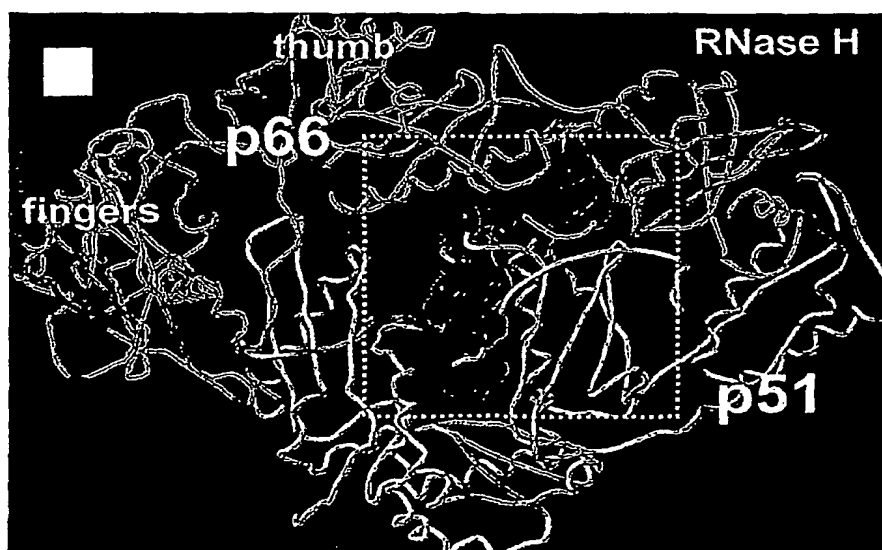


FIG.10B

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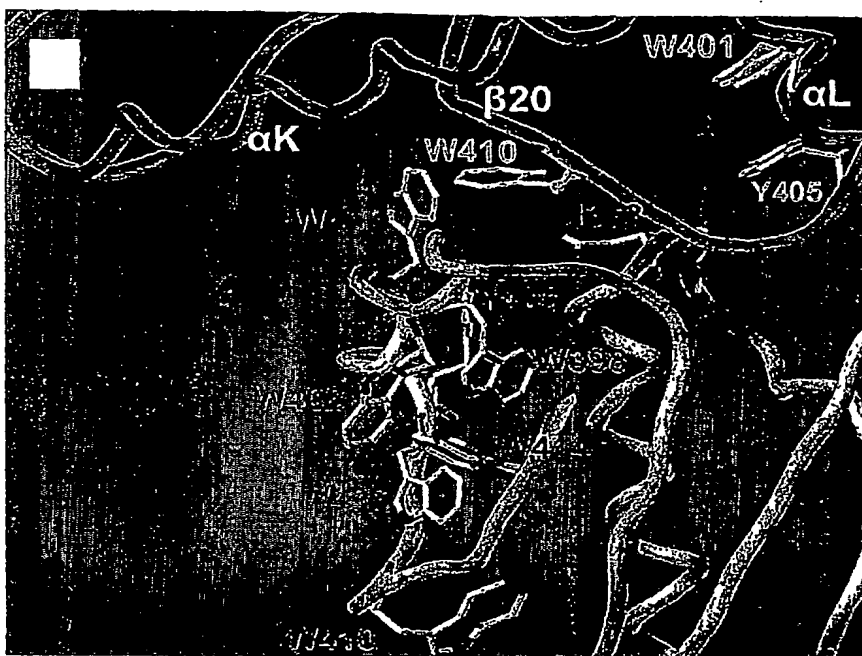


FIG.10C

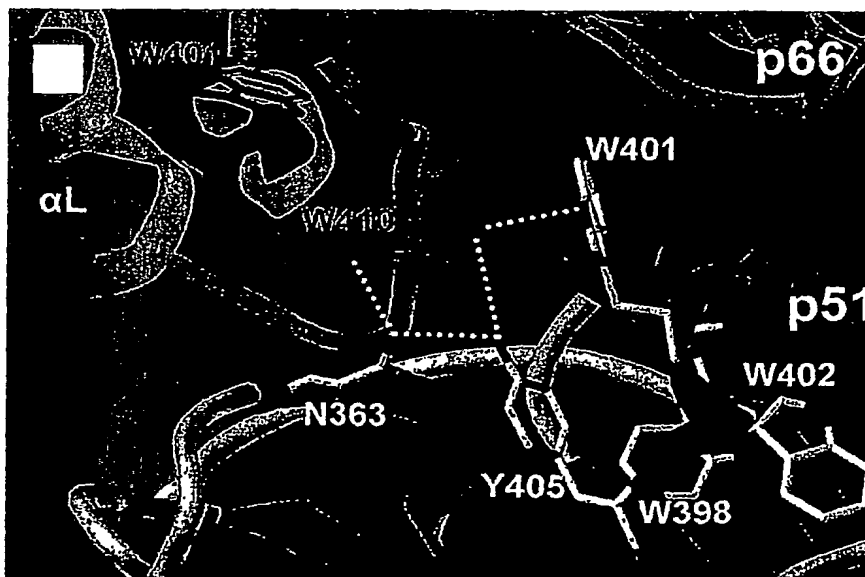


FIG.10D

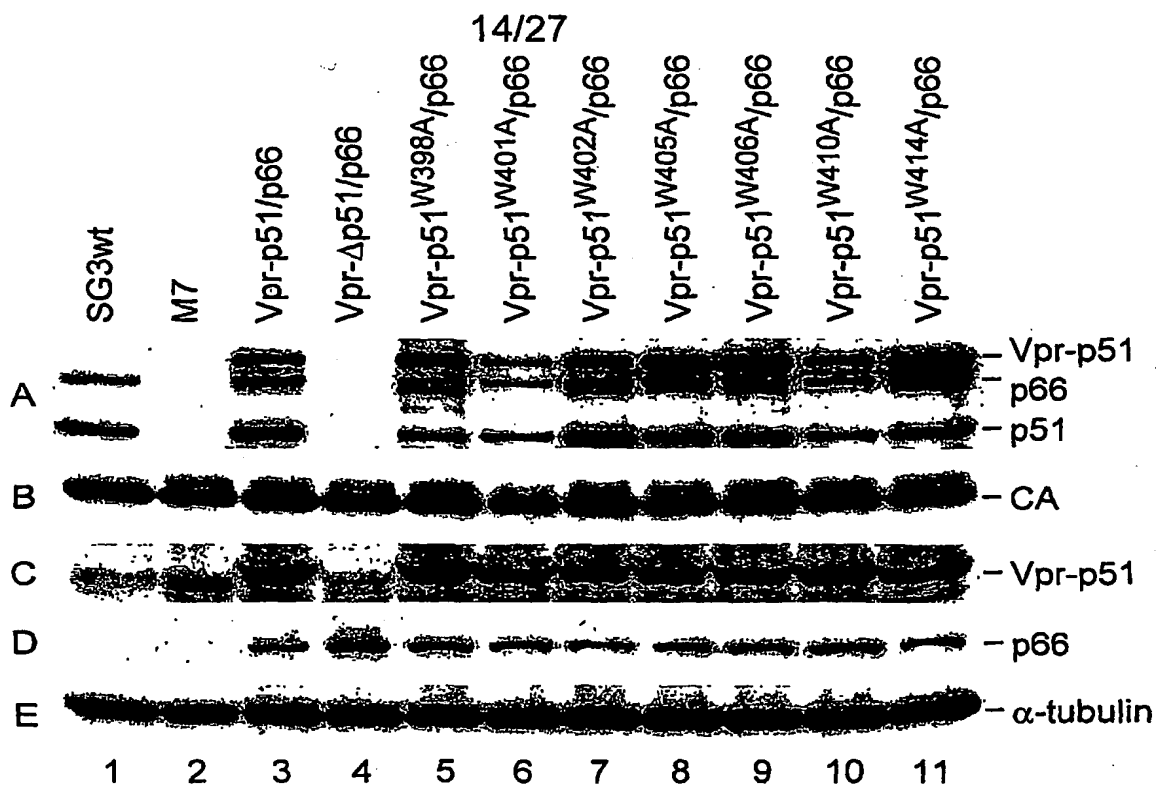
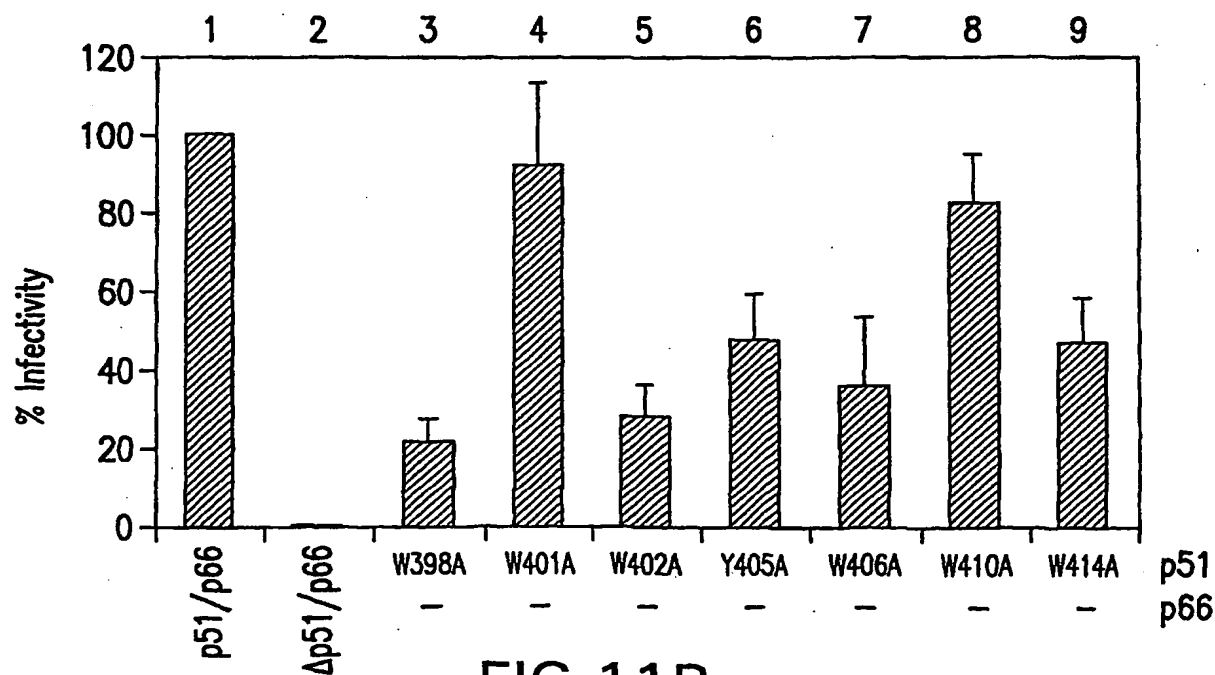
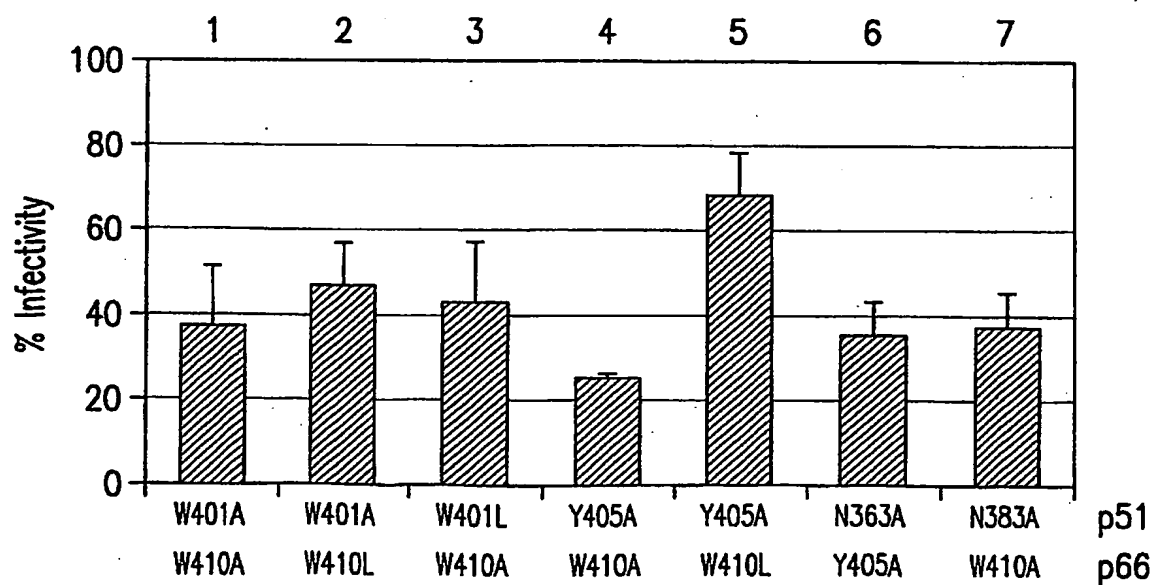
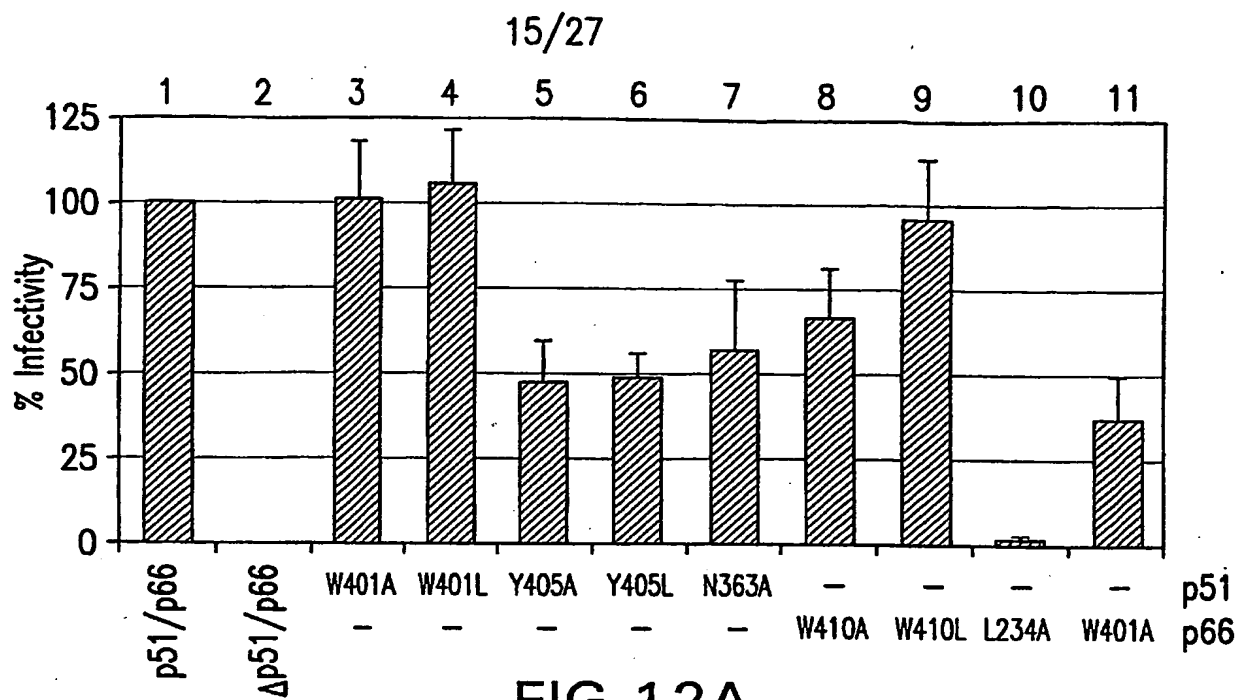


FIG. 11A





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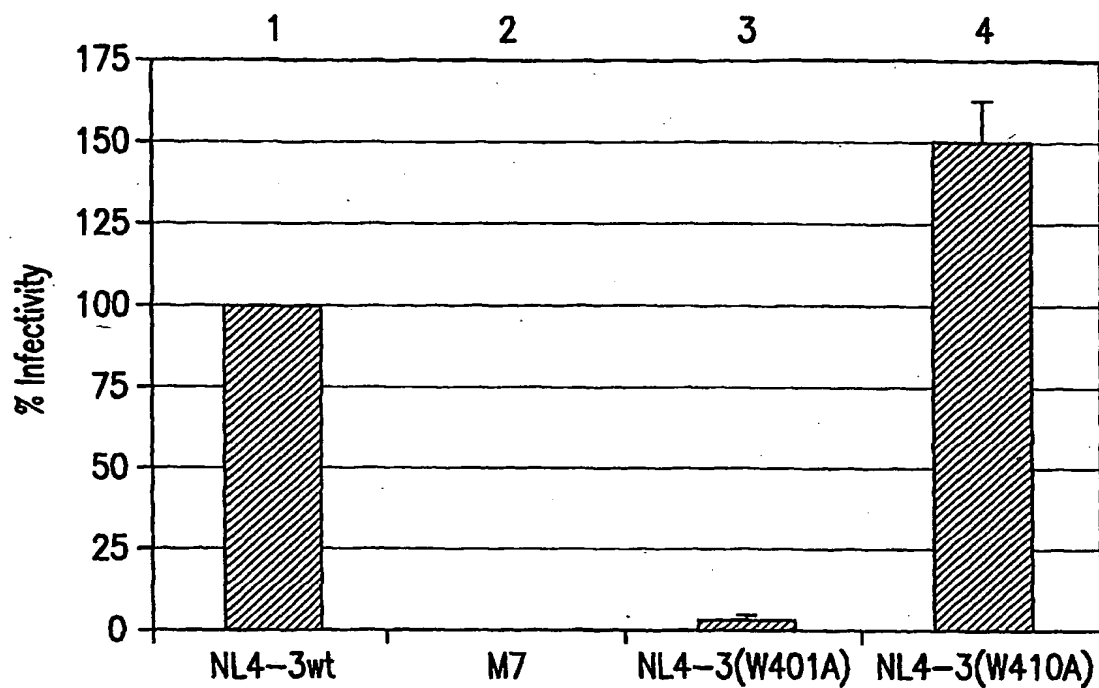


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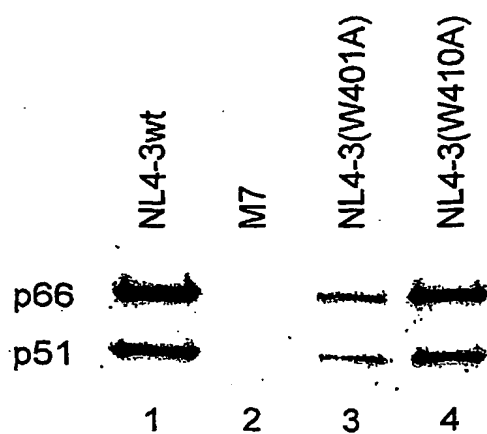


FIG.13B

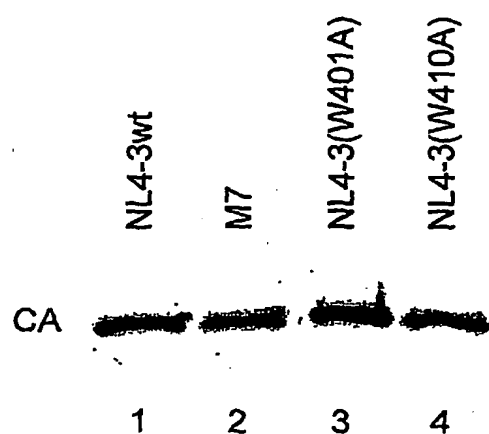


FIG.13C

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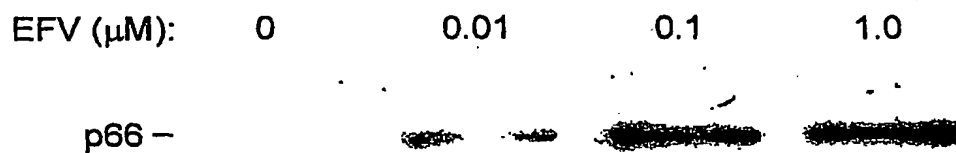


FIG.14A

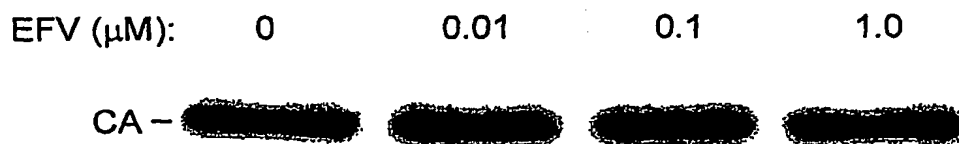


FIG.14B

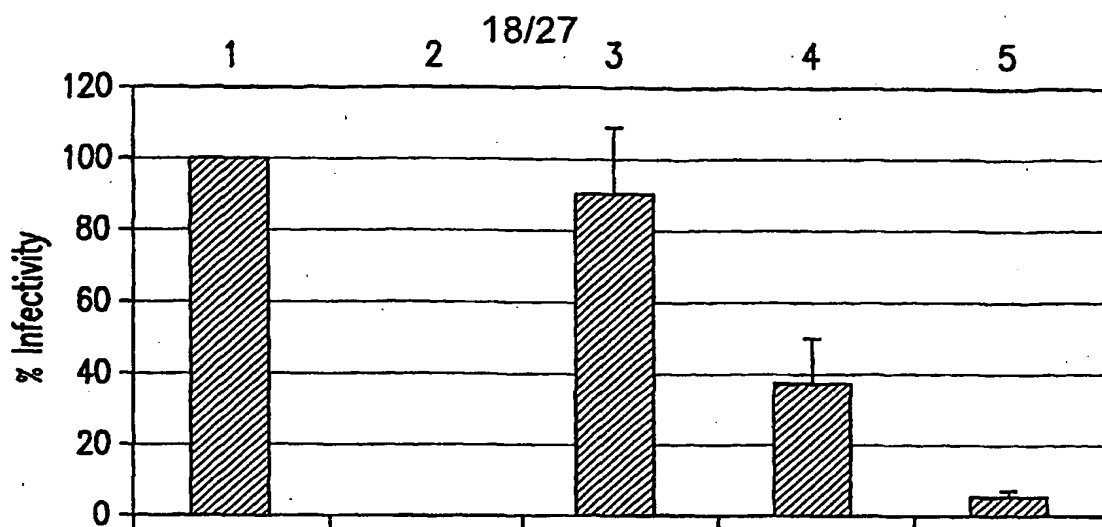


FIG.15A

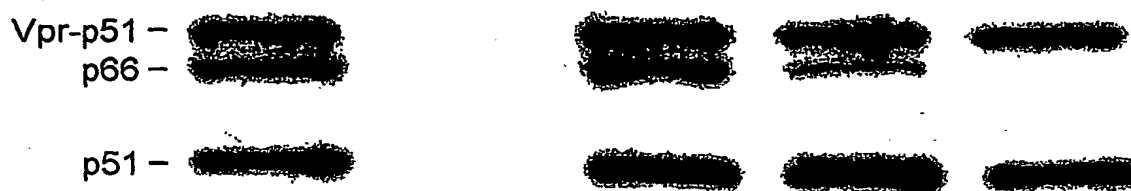


FIG.15B

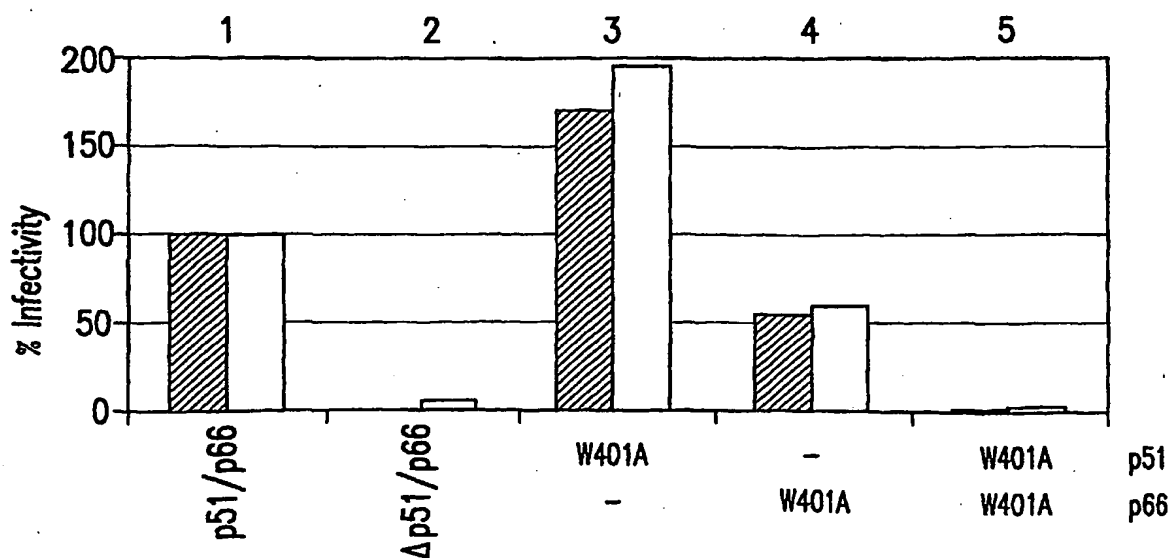


FIG.15C

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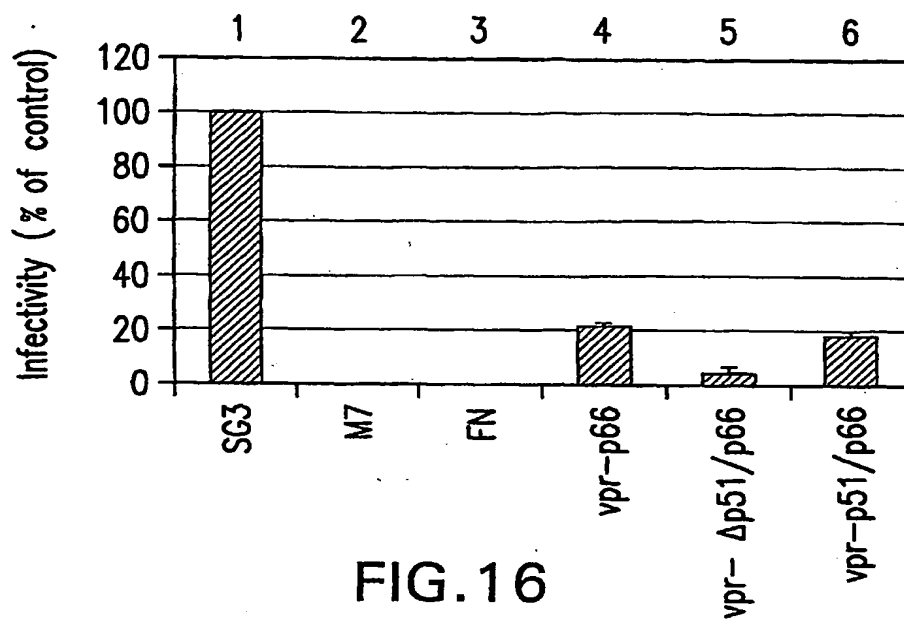


FIG. 16

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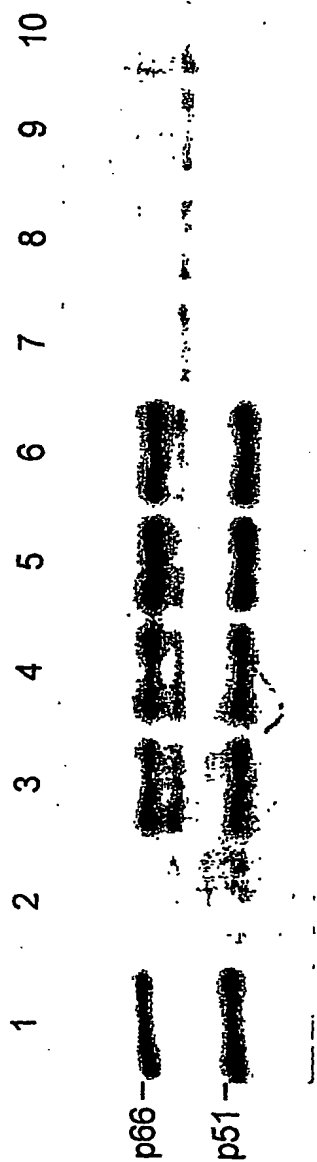


FIG. 17A

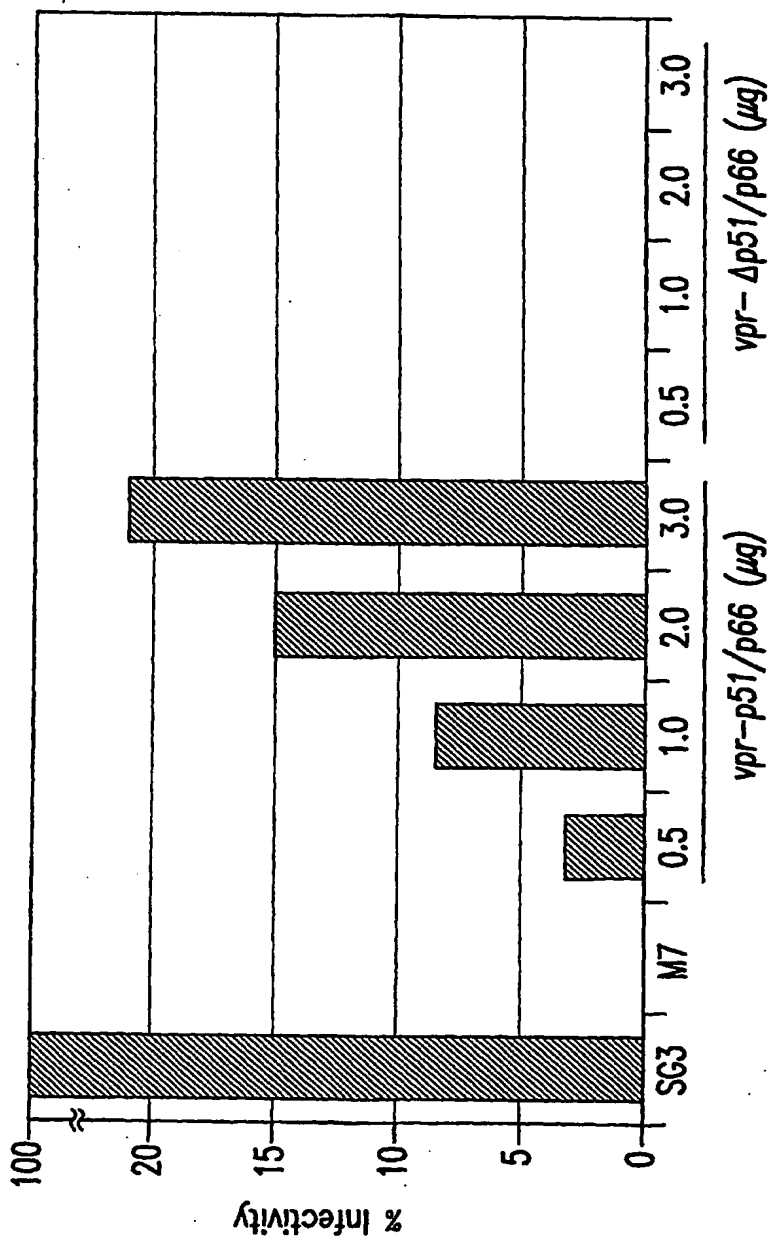


FIG. 17B

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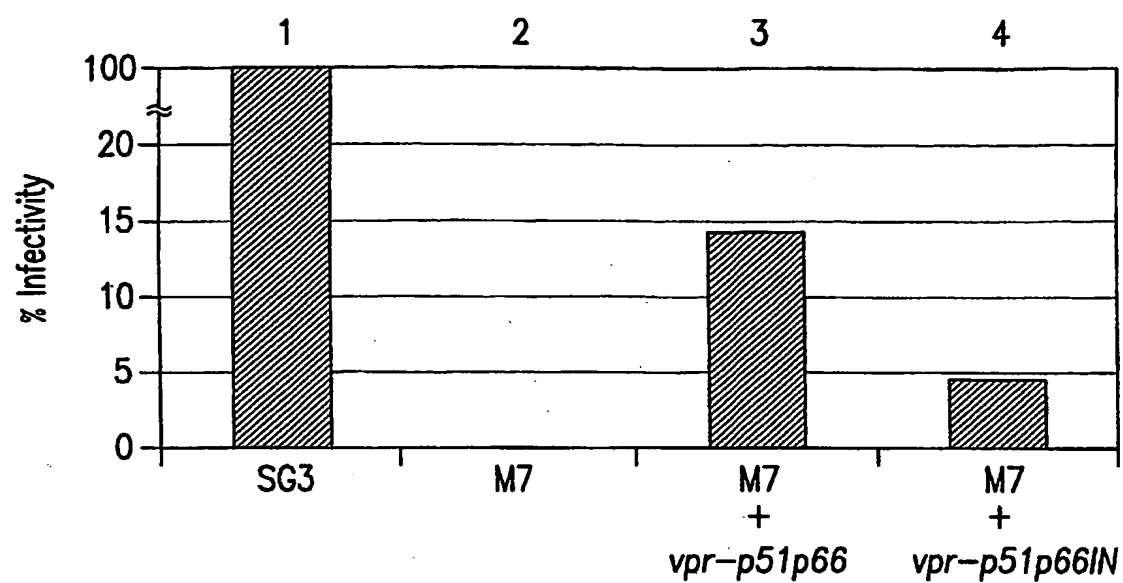


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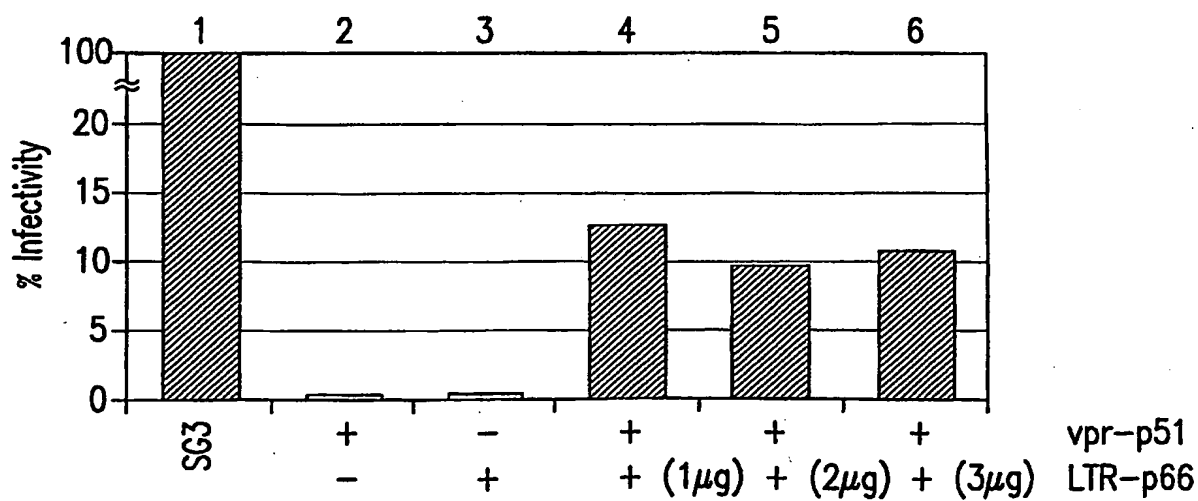


FIG. 18D

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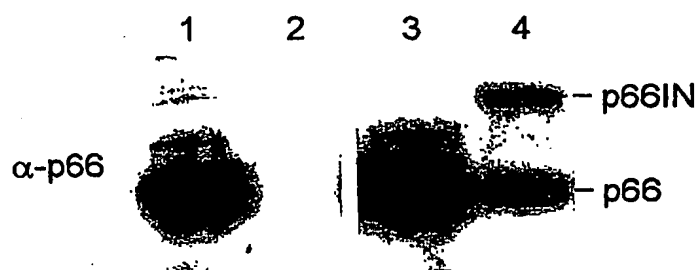


FIG.18B

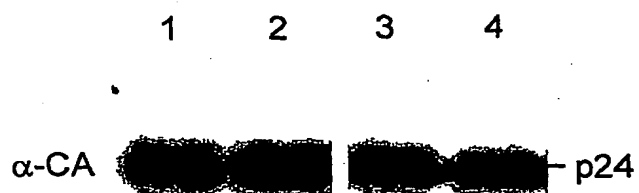


FIG.18C

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FIG. 19A

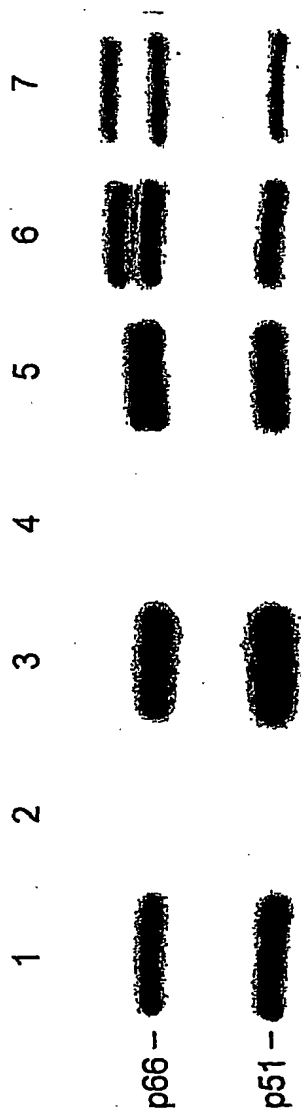
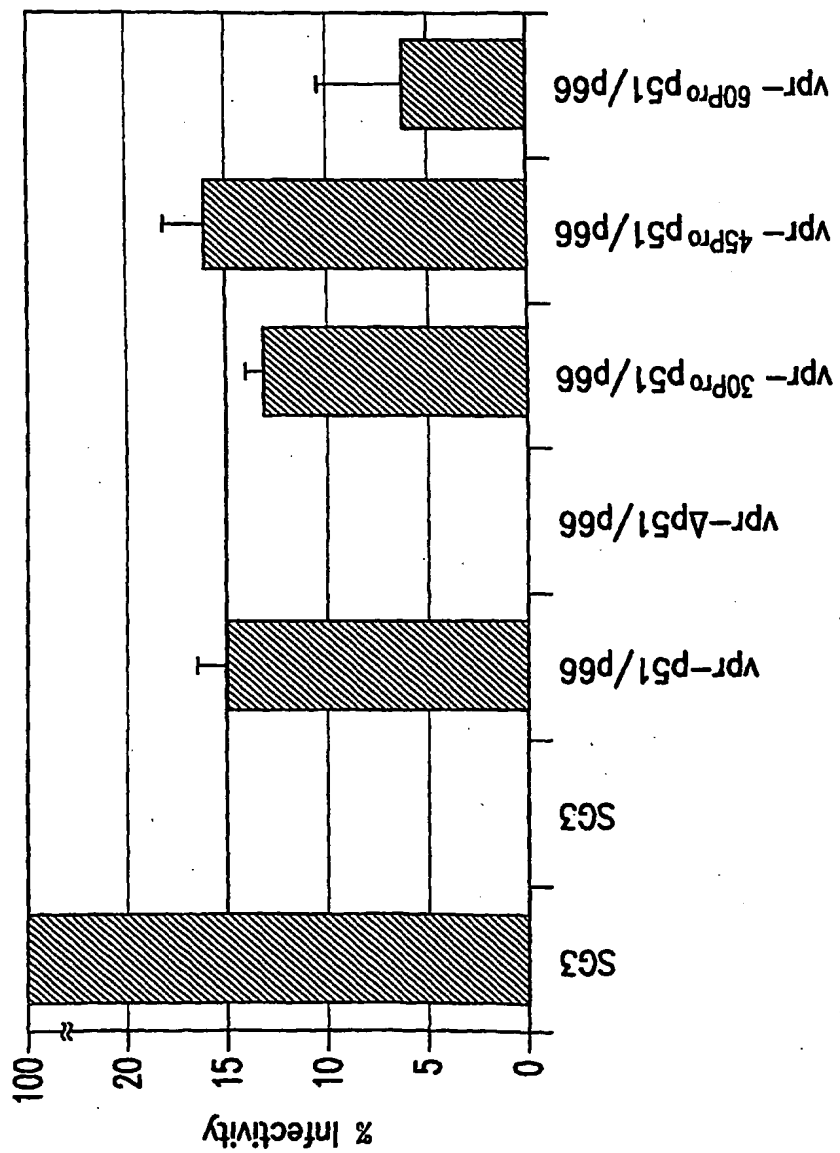


FIG. 19B



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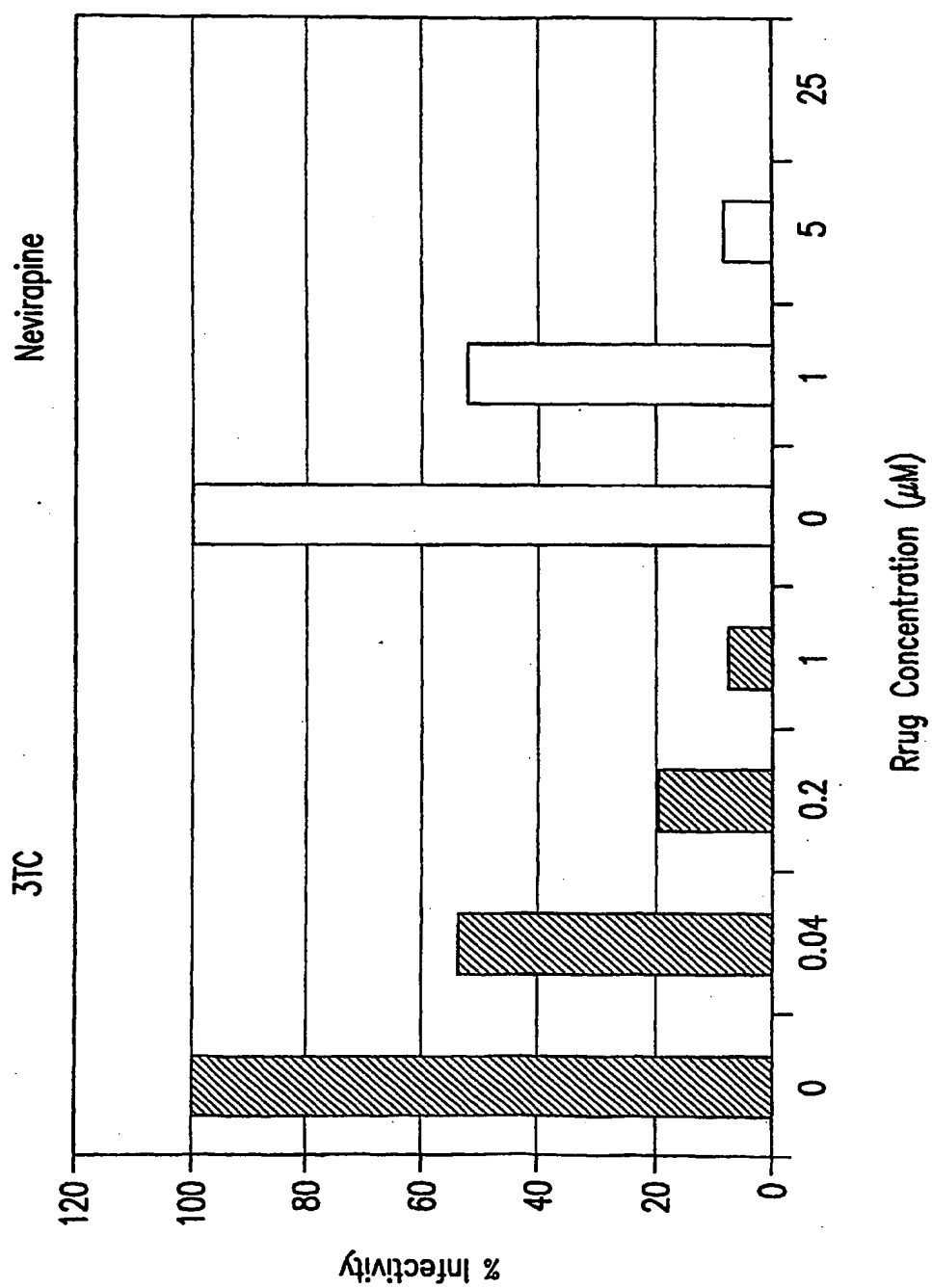


FIG.20

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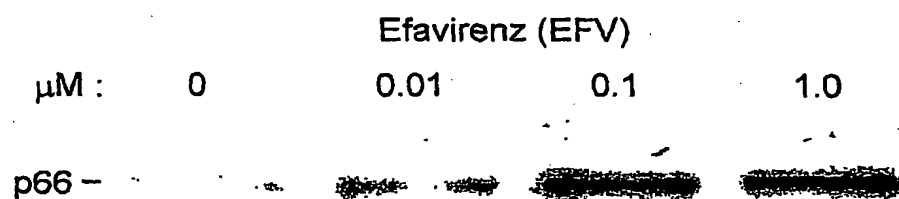


FIG.21A

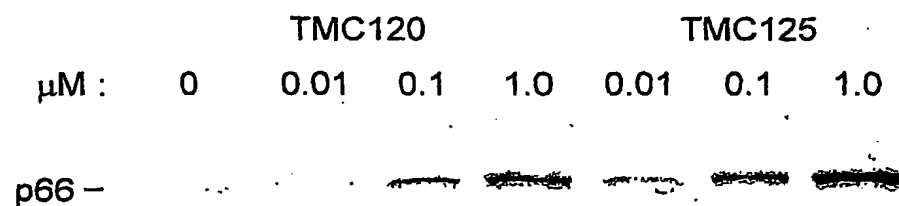


FIG.21B

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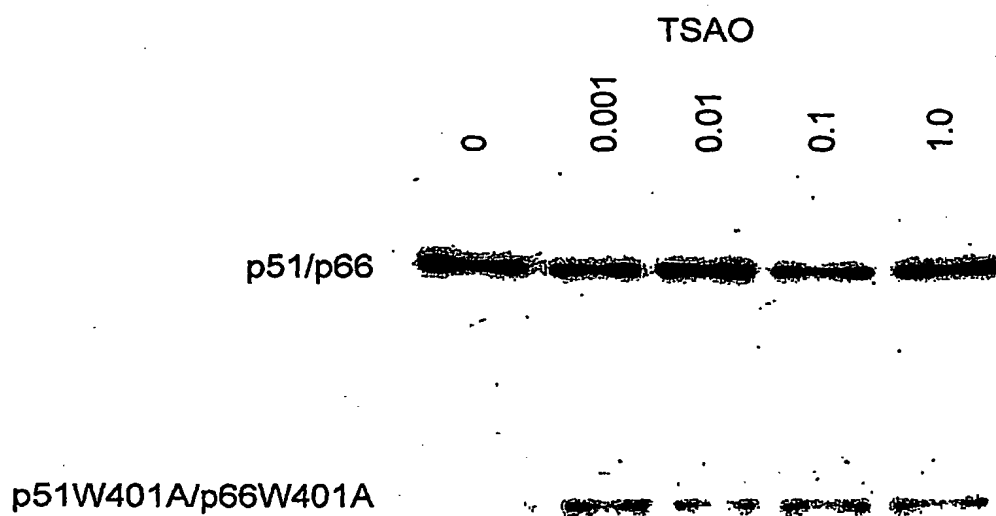


FIG.22

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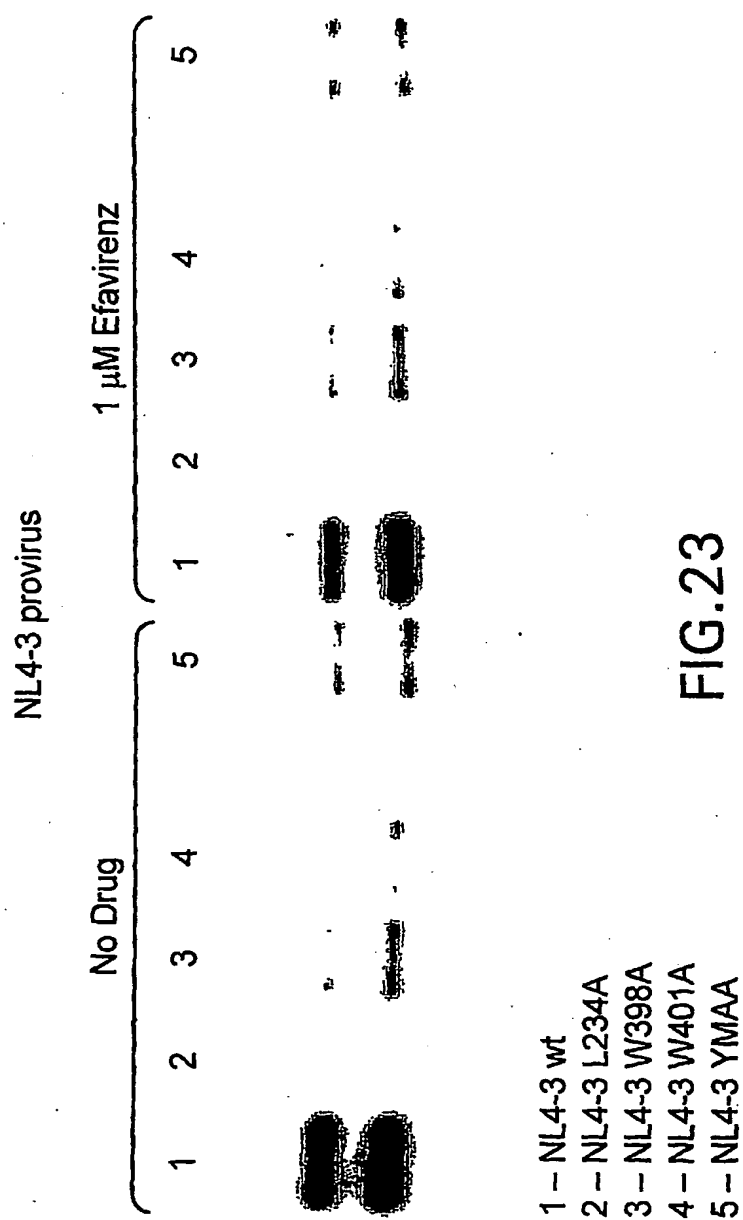


FIG.23

SEQUENCE LISTING

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KAPPES, John C.
 MULKY, Alok
 WU, Xiaoyun

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His Phe Pro Arg Val Trp Leu His Gly Leu Gly Gln His Ile Tyr Glu
      35           40           45
Thr Tyr Gly Asp Thr Trp Ala Gly Val Glu Ala Ile Ile Arg Ile Leu
      50           55           60
Gln Gln Leu Leu Phe Ile His Phe Arg Ile Gly Cys Gln His Ser Arg
65           70           75           80
Ile Gly Ile Thr Arg Gln Arg Arg Ala Arg Asn Gly Ala Ser Arg Ser
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gcgtggaggg catcatcagg atcctgcagc agctgctgtt catccacttc aggatcggct      240
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      20           25           30
Ala Leu Val Glu Ile Cys Thr Glu Met Glu Lys Glu Gly Lys Ile Ser
      35           40           45
Lys Ile Gly Pro Glu Asn Pro Tyr Asn Thr Pro Val Phe Ala Ile Lys
      50           55           60
Lys Lys Asp Ser Thr Lys Trp Arg Lys Leu Val Asp Phe Arg Glu Leu
65           70           75           80
Asn Lys Arg Thr Gln Asp Phe Trp Glu Val Gln Leu Gly Ile Pro His
      85           90           95
Pro Ala Gly Leu Lys Lys Lys Lys Ser Val Thr Val Leu Asp Val Gly
      100          105          110
Asp Ala Tyr Phe Ser Val Pro Leu Asp Glu Asp Phe Arg Lys Tyr Thr
      115          120          125
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Ala Phe Thr Ile Pro Ser Ile Asn Asn Glu Thr Pro Gly Ile Arg Tyr
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 Gln Tyr Asn Val Leu Pro Gln Gly Trp Lys Gly Ser Pro Ala Ile Phe
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 Gln Ser Ser Met Thr Lys Ile Leu Glu Pro Phe Arg Lys Gln Asn Pro
 165 170 175
 Asp Ile Val Ile Tyr Gln Tyr Met Asp Asp Leu Tyr Val Gly Ser Asp
 180 185 190
 Leu Glu Ile Gly Gln His Arg Thr Lys Ile Glu Glu Leu Arg Gln His
 195 200 205
 Leu Leu Arg Trp Gly Leu Thr Thr Pro Asp Lys Lys His Gln Lys Glu
 210 215 220
 Pro Pro Phe Leu Trp Met Gly Tyr Glu Leu His Pro Asp Lys Trp Thr
 225 230 235 240
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 Ile Gln Lys Leu Val Gly Lys Leu Asn Trp Ala Ser Gln Ile Tyr Pro
 260 265 270
 Gly Ile Lys Val Arg Gln Leu Cys Lys Leu Leu Arg Gly Thr Lys Ala
 275 280 285
 Leu Thr Glu Val Ile Pro Leu Thr Glu Glu Ala Glu Leu Glu Leu Ala
 290 295 300
 Glu Asn Arg Glu Ile Leu Lys Glu Pro Val His Gly Val Tyr Tyr Asp
 305 310 315 320
 Pro Ser Lys Asp Leu Ile Ala Glu Ile Gln Lys Gln Gly Gln Gly Gln
 325 330 335
 Trp Thr Tyr Gln Ile Tyr Gln Glu Pro Phe Lys Asn Leu Lys Thr Gly
 340 345 350
 Lys Tyr Ala Arg Met Arg Gly Ala His Thr Asn Asp Val Lys Gln Leu
 355 360 365
 Thr Glu Ala Val Gln Lys Ile Thr Thr Glu Ser Ile Val Ile Trp Gly
 370 375 380
 Lys Thr Pro Lys Phe Lys Leu Pro Ile Gln Lys Glu Thr Trp Glu Thr
 385 390 395 400
 Trp Trp Thr Glu Tyr Trp Gln Ala Thr Trp Ile Pro Glu Trp Glu Phe
 405 410 415
 Val Asn Thr Pro Pro Leu Val Lys Leu Trp Tyr Gln Leu Glu Lys Glu
 420 425 430
 Pro Ile Val Gly Ala Glu Thr Phe
 435 440

<210> 5

<211> 440

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 5

Pro Ile Ser Pro Ile Glu Thr Val Pro Val Lys Leu Lys Pro Gly Met
 1 5 10 15
 Asp Gly Pro Lys Val Lys Gln Trp Pro Leu Thr Glu Glu Lys Ile Lys
 20 25 30
 Ala Leu Val Glu Ile Cys Thr Glu Met Glu Lys Glu Gly Lys Ile Ser
 35 40 45
 Lys Ile Gly Pro Glu Asn Pro Tyr Asn Thr Pro Val Phe Ala Ile Lys
 50 55 60
 Lys Lys Asp Ser Thr Lys Trp Arg Lys Leu Val Asp Phe Arg Glu Leu
 65 70 75 80

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Asn Lys Arg Thr Gln Asp Phe Trp Glu Val Gln Leu Gly Ile Pro His
      85                      90                      95
Pro Ala Gly Leu Lys Lys Lys Lys Ser Val Thr Val Leu Asp Val Gly
      100                    105                    110
Asp Ala Tyr Phe Ser Val Pro Leu Asp Glu Asp Phe Arg Lys Tyr Thr
      115                    120                    125
Ala Phe Thr Ile Pro Ser Ile Asn Asn Glu Thr Pro Gly Ile Arg Tyr
      130                    135                    140
Gln Tyr Asn Val Leu Pro Gln Gly Trp Lys Gly Ser Pro Ala Ile Phe
      145                    150                    155                    160
Gln Ser Ser Met Thr Lys Ile Leu Glu Pro Phe Arg Lys Gln Asn Pro
      165                    170                    175
Asp Ile Val Ile Tyr Gln Tyr Met Asp Asp Leu Tyr Val Gly Ser Asp
      180                    185                    190
Leu Glu Ile Gly Gln His Arg Thr Lys Ile Glu Glu Leu Arg Gln His
      195                    200                    205
Leu Leu Arg Trp Gly Leu Thr Thr Pro Asp Lys Lys His Gln Lys Glu
      210                    215                    220
Pro Pro Phe Leu Trp Met Gly Tyr Glu Leu His Pro Asp Lys Trp Thr
      225                    230                    235                    240
Val Gln Pro Ile Val Leu Pro Glu Lys Asp Ser Trp Thr Val Asn Asp
      245                    250                    255
Ile Gln Lys Leu Val Gly Lys Leu Asn Trp Ala Ser Gln Ile Tyr Pro
      260                    265                    270
Gly Ile Lys Val Arg Gln Leu Cys Lys Leu Leu Arg Gly Thr Lys Ala
      275                    280                    285
Leu Thr Glu Val Ile Pro Leu Thr Glu Glu Ala Glu Leu Glu Leu Ala
      290                    295                    300
Glu Asn Arg Glu Ile Leu Lys Glu Pro Val His Gly Val Tyr Tyr Asp
      305                    310                    315                    320
Pro Ser Lys Asp Leu Ile Ala Glu Ile Gln Lys Gln Gly Gln Gly Gln
      325                    330                    335
Trp Thr Tyr Gln Ile Tyr Gln Glu Pro Phe Lys Asn Leu Lys Thr Gly
      340                    345                    350
Lys Tyr Ala Arg Met Arg Gly Ala His Thr Asn Asp Val Lys Gln Leu
      355                    360                    365
Thr Glu Ala Val Gln Lys Ile Thr Thr Glu Ser Ile Val Ile Trp Gly
      370                    375                    380
Lys Thr Pro Lys Phe Lys Leu Pro Ile Gln Lys Glu Thr Trp Glu Thr
      385                    390                    395                    400
Trp Trp Thr Glu Tyr Trp Gln Ala Thr Trp Ile Pro Glu Trp Glu Phe
      405                    410                    415
Val Asn Thr Pro Pro Leu Val Lys Leu Trp Tyr Gln Leu Glu Lys Glu
      420                    425                    430
Pro Ile Val Gly Ala Glu Thr Phe
      435                    440

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<210> 6

<211> 170

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 6

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Lys Glu Gly His Gln Met Lys Glu Cys Thr Glu Arg Gln Ala Asn Phe
  1                      5                      10                      15
Leu Gly Lys Ile Trp Pro Ser His Lys Gly Arg Pro Gly Asn Phe Leu
  20                      25                      30

```

Gln Ser Arg Pro Glu Pro Thr Ala Pro Pro Glu Glu Ser Phe Arg Cys
 35 40 45
 Gly Glu Glu Lys Thr Thr Pro Pro Gln Lys Pro Glu Gln Thr Asp Lys
 50 55 60
 Glu Leu Tyr Pro Leu Ala Ser Leu Arg Ser Leu Phe Gly Gln Arg Pro
 65 70 75 80
 Leu Val Thr Ile Lys Ile Gly Gly Gln Leu Lys Glu Ala Leu Leu Asp
 85 90 95
 Thr Gly Ala Asp Asp Thr Val Leu Glu Asp Met Ser Leu Pro Gly Lys
 100 105 110
 Trp Lys Pro Lys Met Ile Gly Gly Ile Gly Gly Phe Ile Lys Val Arg
 115 120 125
 Gln Tyr Asp Gln Ile Pro Ile Glu Ile Cys Gly His Lys Ala Ile Gly
 130 135 140
 Thr Val Leu Ile Gly Pro Thr Pro Val Asn Ile Ile Gly Arg Asn Leu
 145 150 155 160
 Leu Thr Gln Ile Gly Cys Thr Leu Asn Phe
 165 170

<210> 7

<211> 511

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 7

aaaggaagga	caccaaata	aagaatgcac	tgagagacag	gctaattttt	tagggaaaat	60
ctggccttcc	cacaagggaa	ggccagggaa	ctttctccag	agcagaccag	agccaacagc	120
cccaccagaa	gagagcttca	ggtgtgggga	ggagaaaaca	actccccctc	agaagccgga	180
gcagacagac	aaggaactgt	atccttttagc	ttccctcaga	tcactctttg	gcaacgaccc	240
ctcgctcaca	taaagatagg	ggggcagcta	aaggaagctc	tattagatac	aggagcagat	300
gatacagtat	tagaagacat	gagtttgcca	ggaaaatgga	agccaaaaat	gataggggga	360
attggaggtt	ttatcaaagt	aagacagtat	gatcagatac	ctatagaaat	ctgtggggcat	420
aaagctatag	gtacagtatt	aataggacca	acacctgtca	acataattgg	aagaaatctg	480
ttgacacaga	ttggttgcac	tttaaatttt	c			511

<210> 8

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 8

Tyr Met Asp Asp
 1

<210> 9

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<220>
<221> VARIANT
<222> 2
<223> Xaa = any amino acid

<400> 9
Tyr Xaa Asp Asp
1

<210> 10
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 10
aagcccggga tggatggccc aaaagt

26

<210> 11
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 11
tcctaaacgc gtctccctct aagctgctca atttacttag aaagt

45

<210> 12
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 12
actttctaag taaattgagc agcttagagg gagacgcgtt tagga

45

<210> 13
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 13
tatgtcgaca cccaattatg aaaag

25

<210> 14
<211> 32
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 14

tagatcagat ctgttgactc agattgggtg ca

32

<210> 15

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 15

atctacacgc'gtttagaagg tttctgcgcc tt

32

<210> 16

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 16

ttattaacgc gtccgcccct ctcctccccc cc

32

<210> 17

<211> 69

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 17

ccatcccggg ctttaatttt actggtacag tttcaatagg actaatgggt cccatggtat
tatcgtctt

60

69

<210> 18

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 18

agcttgccctt gagtgcttca a

21

<210> 19

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 19

ctgctagaga ttttccacac tgacta

26

<210> 20

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 20

ggctagctag ggaacccact g

21

<210> 21

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; note =
synthetic construct

<400> 21

atactgacgc tctcgaccc at

22